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The International Workshop on Anthrax held in King Alfred's College, Winchester, England, 19-21 September 1995 was attended by 124 individuals representing 22 countries. Inestimable value resulted from exchanges of experience among participants associated with *Bacillus anthracis* from widely differing standpoints, ranging from those who encounter the disease on a regular basis in Africa, India, Indonesia, and Turkey, to those in Europe and the USA who study intricate molecular facets of the organism without having ever seen the disease in humans or animals.

The proceedings of the workshop were received from the printer on 17 June 1996 and 3 copies were sent (by courier on that same date) to the USAMRMC as stipulated in the Grant agreement. The volume contains 81 papers by authors from 22 countries and represents the most comprehensive coverage available today of the current incidence and importance of anthrax and of research into its pathogenesis, molecular biology and control.

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Grant DAMD17-95-1-5035 was awarded specifically to "defray the cost associated with conducting the Internatio Workshop on Anthrax, 19-21 September 1995". The stipulations listed above which were relevant to this project were adhered to within the limits of the powers of the Principal Investigator in his capacities as Convener o the Workshop and Editor of the Proceedings.

Peter C.B. Turnbull 6 August 199

PI - Signature Date

Peter C.B. Turnbull, PhD

SALISBURY MEDICAL BULLETIN

Proceedings of the
International Workshop on

ANTHRAX

Winchester, England, September 19-21, 1995

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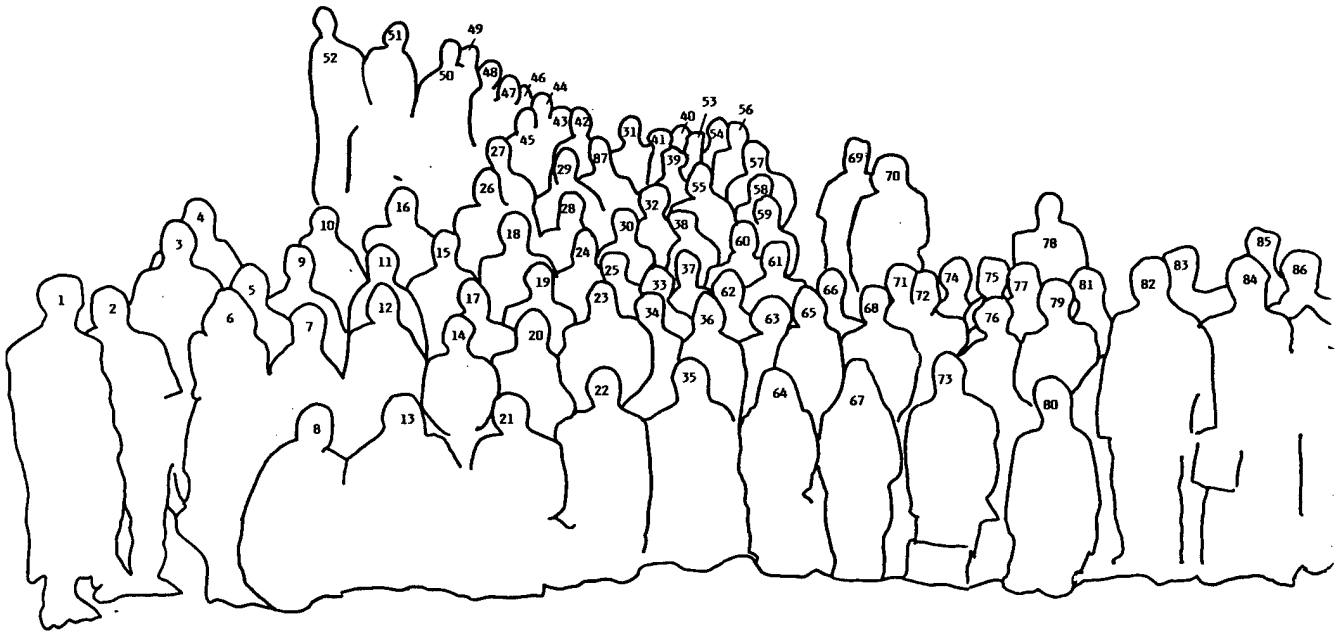
Group photograph at the Workshop



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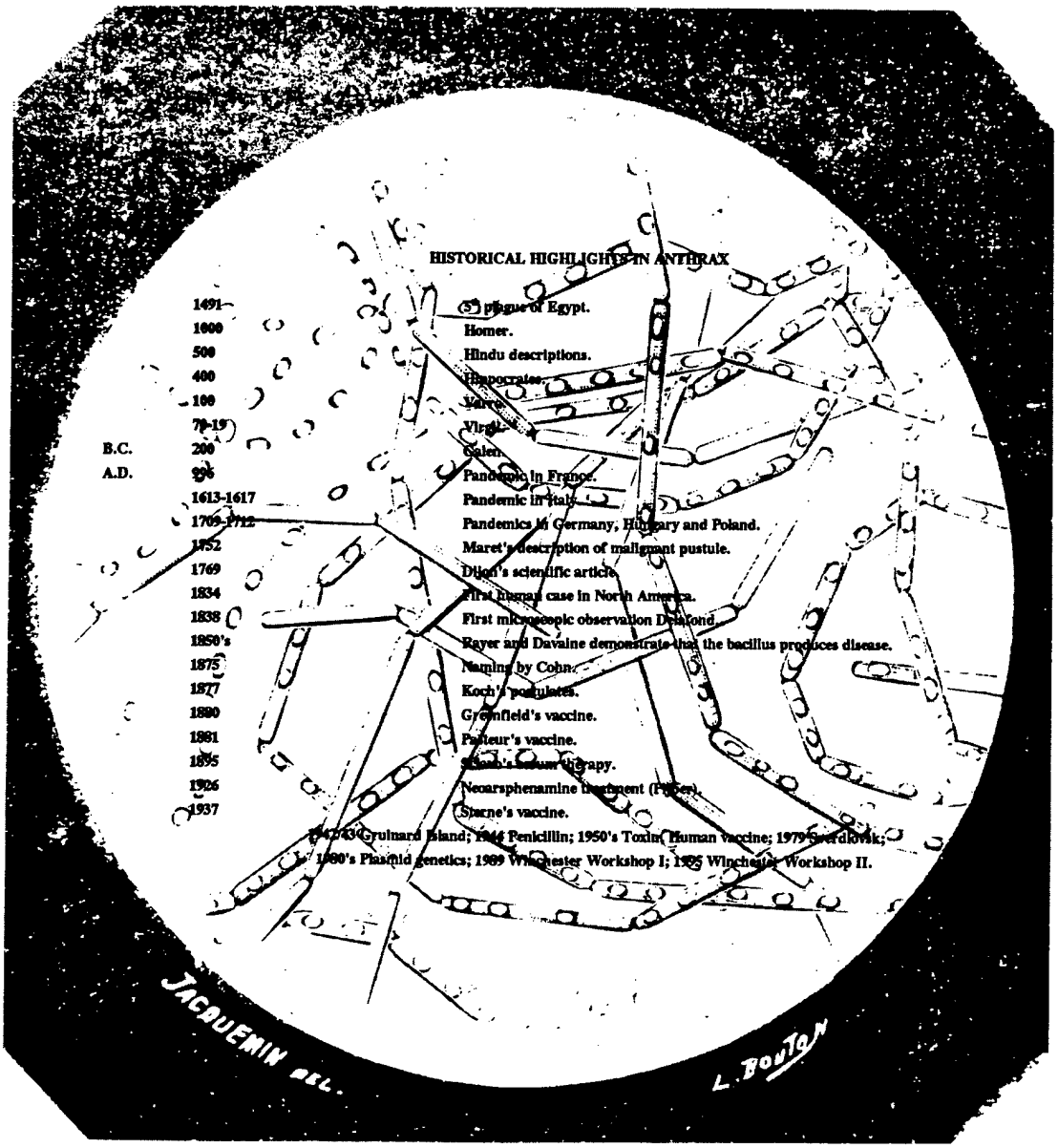
Front cover of Workshop Manual



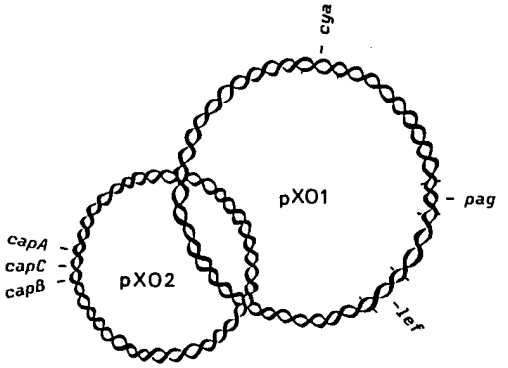
INTERNATIONAL WORKSHOP

ON

ANTHRAX

19 - 21 SEPTEMBER 1995

WINCHESTER, ENGLAND



The theme, in harmony with l'Année Pasteur, was anthrax from historical times to the present day. The background shows the view through Louis Pasteur's microscope of a chicken broth culture of *Bacillus anthracis* after several days (Figure 4 of Chamberland's book "Le Charbon et la Vaccination Charbonneuse d'après les Travaux Récents de M Pasteur" (1883).

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EDITOR'S NOTE

The fundamental purpose of the International Workshop on Anthrax was to highlight current concerns and interests relating to anthrax, and the work being carried out on the disease or its causative agent around the world. Consequently, although the papers submitted for inclusion in these proceedings were sent to reviewers, this was done with an accompanying instruction that that the purpose of doing so was not reject any scripts, but to raise standards as far as possible where necessary.

In a number of instances, it has to be said that the presentation of data, or conclusions therefrom, in a small proportion of the papers left the reviewers uneasy and readers are urged to view papers in this volume critically, particularly if citing them in their own reports.

Unfortunately, as a result of conflicting pressures, it was not possible to standardise the style of references throughout the volume without causing excessive delay and incurring untoward costs. However, with just one or two exceptions, one generally accepted style or another has been conformed to in each paper.

The editor is grateful to the reviewers for their time and help.

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Risks and hazards

Pathogenesis and molecular biology

Prophylaxis and control

World situation 1993/94

M.E. HUGH-JONES

D/WHO Collaborating Center, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA

In a phrase, the more things change, the more they stay the same. While there have been some successes, as in Indonesia and Zimbabwe, these have been balanced by failures and zero-progress elsewhere. In too many countries livestock anthrax is diagnosed clinically without laboratory confirmation. This is a result of professional and bureaucratic laziness; of an unfounded fear of the disease itself; of the firm belief that if reality is ignored it does not exist; of the 'importance' of anthrax in cattle while totally ignoring its economic and nutritional significance when in small ruminants and pigs (worldwide, cattle are kept by men while sheep and goats by women and children); and the false belief that nothing can be done seriously to control it because the ground is eternally contaminated. While there is clear evidence and experience demonstrating that this disease can be eradicated from livestock, the pervasive veterinary attitude is that it is a permanent hazard. One of the results of these factors is that the surveillance and reporting of this disease is defective in many countries at all levels of development, starting with the USA. If this is to change, the veterinary authorities must first treat this disease seriously and as one that can have a true zero-incidence, at least in livestock.

Taking the continents in turn:

In North America the endemic situation in wood buffalo in northern Alberta and the North West Territories continues; this was first noted in 1963. It is under active investigation. It is characterised by being limited almost entirely to sexually active males and starts about three weeks before the rutting season and ceases with the first frost in the Autumn. While tabanid flies and corvids will play some part in the spread of infection, which is non-focal, this ongoing epizootic raises a series of fundamental questions of latent infections, sporulation, and whether this is a special Arctic 'strain'. There is a widespread but sporadic incidence in the western Canadian prairies of Alberta & Saskatchewan that affects grazing cattle. This started some years ago with cases in December and January, which would suggest a contaminated feed-stuff origin. In the USA there appear to be two endemic areas, (1) in NW Mississippi and adjoining SE Arkansas affecting mainly livestock and with tabanid involvement, and (2) in western Texas affecting sheep and white-tailed deer, and carcass contamination; similar cases are reported in the adjoining parts of Mexico. Within Mexico, reported bovine cases are widespread with less than half laboratory confirmed; sheep cases are under-reported. In all three countries, the pattern of known livestock cases nationally would suggest that anthrax carcasses are entering the feed cycle.

In Central America the disease is epidemic in Guatemala; Honduras has recently reported cases; and Panama, though not reporting cases, is probably affected. With the exception of Belize, it is a serious problem in Central America. It is absent from the Caribbean, excepting Haiti; the epidemic state of that next door country must make one suspicious of the freedom claimed by the Dominican Republic. In South America, Chile reports that the disease is endemic; this may reflect a strengthened and improved veterinary service surveillance more than a true change in incidence. Brazil, which claimed not to have had anthrax for many years while still carrying out significant annual vaccination, has recently begun reporting cases though they have yet to be laboratory confirmed. On the other hand, Uruguay has just reported its first anthrax-free year.

In Europe, the major afflicted regions remain Turkey, Greece, Albania, southern Italy, Romania, and central Spain. If Portugal were to cease vaccinating (238,700 animals were vaccinated in 1994) one would know whether their officially claimed absence of cases was real. Elsewhere, cases are sporadic. The fighting in the Balkans obscures the reality there but the disease is probably significantly reduced; it is in the tropics and sub-tropics that civil wars increase the incidence. The pattern of cases in Norway, a country essentially without anthrax over many years, suggests a feedborne epidemiology.

Africa remains severely afflicted with livestock and human anthrax, especially in West Africa, compounded by civil strife over many years. South Africa, essentially all but free, suffers from truly sporadic outbreaks and large numbers of deaths in affected flocks presumably because of diagnostic delays. I am unimpressed by official claims of disease-freedom by Egypt and Malawi and await hard proof of this beyond reports that the diagnostic laboratories have not confirmed a case.

The traditional anthrax belt from Turkey to Pakistan remains severely affected. In 1994 Syria reported an epidemic status for sheep and goats. The Caucasus and Transcaucasus states must be included in this anthrax belt. If India broke down their reports by states, the belt would probably connect up with the anthrax problems in Southeast Asia, where it frequently affects water buffalo and pigs. Sri Lanka has not seen a laboratory confirmed case in decades but they are not receiving samples from the field. Their old, traditionally affected area is in the Tamil north of the island where there are severe civil problems. With help from the Australian government, anthrax is now a localised problem in Indonesia. Australia, with a traditional small affected area in New South Wales, has recently had cases in Western Australia. The Korean Republic, which had not had reported a case since 1978, had two affected cattle and 28 human cases in 1994. China has only recently started to report livestock diseases internationally. In 1993 they reported 650 affected cattle and 150 in 1994, with no cases reported in other livestock. With 1654 human cases in 1993 but only 15 in 1994, it is obvious that the true picture is still obscure and maybe not as reported. Public health reports indicate that the disease exists in the poor peripheral provinces of that country.

Overall, there is a persuasive, global epidemiological pattern of contaminated animal feedstuffs. By this I mean not just the traditional English model resulting from imported sun-dried hides and bones, now very significantly and successfully controlled in the UK, but from the unmonitored local use of dead stock going for meat and bone meals. This will only be controlled when sudden, unexplained livestock deaths are routinely checked. Similarly, governments must begin structured surveillance programmes for this disease. But, as with many ignored diseases, an improved surveillance immediately always results in more reported cases. While temporarily embarrassing, the better appreciation of reality can pay off in improved and cheaper controls. It should be remembered that an efficient and effective anthrax control programme will, by its very nature, eradicate the disease in livestock in the medium term, Pasteur notwithstanding.

The global situation is summarised in the accompanying figure.

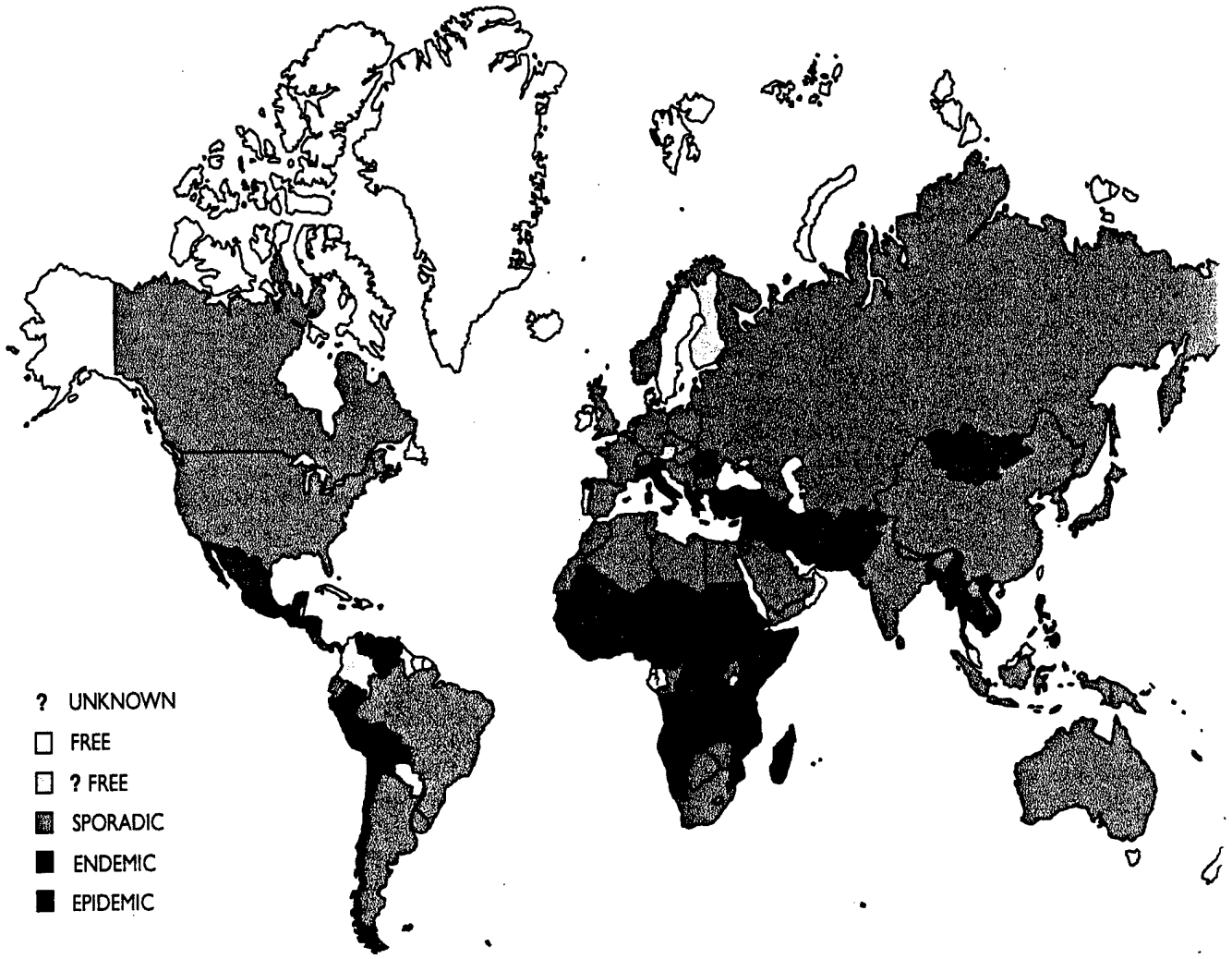

Figure 1. Global livestock anthrax in 1994

Quality of reports - OIE World Animal Health in 1993 and the 1993 FAO-OIE-WHO Animal Health Yearbook

M.E. HUGH-JONES

D/WHO Collaborating Center, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA

One of the ways in which one might judge the seriousness in which countries regard disease surveillance is by the quality of their annual reports to international agencies. If this is the case, few countries appear to take anthrax seriously.

It is impossible to directly compare the reports to OIE with those to FAO, the editors of the yearbook, because the former gives numbers of cases and deaths, when possible, and the latter provides a coded system ... -, +, ++, +++, etc. It is not unusual to find a country reporting an absence of cases to FAO (-) and numbers of anthrax cases to OIE; and *vice-versa.* Similarly, the former may declare that a disease has never occurred ("oooo"), when reference to past yearbooks amply reveals an alternative situation over many years. While the OIE data are comforting in their exactitude there are no common standards as to what represents an anthrax case or death . Too often the data refers to clinical diagnoses without laboratory confirmation; in spite of popular professional belief a clinical diagnosis of anthrax is meaningless because of similarities to other common causes of sudden death in livestock. The cutaneous lesion in humans may be pathognomonic aided by its response to antibiotics but cutaneous lesions in livestock are rare.

To highlight these problems, the livestock population data were abstracted from 78 countries as given in the OIE World Animal Health in 1993 (part 2) report, and compared with the similar data published in the *1993 FAO-OIE-WHO Animal Health Yearbook.* The results are shown in the Tables. In summary, only 52% of the data agreed within an error-bound of 10%. Put another way, 33% of the data varied by more than 20%. These errors were independent of the development of countries.

Where these differences are generated cannot be defined by us at this time. Some were clearly clerical errors (e.g., simple transposition of numbers); some were illogical (e.g., fewer animals than herds or flocks). Some may be the result of different units within the relevant Ministries or Departments of Agriculture reporting to OIE and to FAO. Some may occur in Rome and in Paris. Whichever and whatever the reasons, the result is serious doubt as to how many countries actually know what is going on.

If this disease is to be controlled:

(1) these agencies must foster actively the development of reliable surveillance systems. And frankly, there are few livestock diseases that are easier to accurately monitor than anthrax.

(2) this situation should be brought to the attention of the DG/OIE so that it can be discussed by the member countries' CVOs at their annual meeting in Paris.

Table 1. Agency comparison of population data, 1993. Livestock data from 78 countries.

Country	Bovines		Sheep + Goats		Swine	
	OIE	FAO	OIE	FAO	OIE	FAO
Australia	23,879,989	23,662,000	148,550,797	163,687,000	2,873,390	2,531,000[1]
Austria	2,336,267	2,534,000	380,254	346,000	3,816,221	3,638,000
Bangladesh	23,200,000	23,259,000	**30,793,000**	**23,254,000**	150,000	na[2]
Barbados	**12,587**	**30,000**	**43,509**	**103,000**	**28,897**	**45,000**[3]
Belgium& Luxemburg	3,473,501	3,360,000	191,597	177,000	6,618,101	6,496,000
Benin	1,141,000	1,088,000	**1,484,400**	**1,934,000**	513,400	515,000
Bhutan	**240,604**	**413,000**	**45,519**	**87,000**	**43,703**	**73,000**
Bolivia	5,619,267	5,607,000	9,109,315	8,790,000	2,250,948	2,177,000
Brazil1	44,154,103	152,136,000	31,710,481	32,300,000	33,015,038	34,290,000
Bulgaria	951,618	np[5]	5,425,525	np	2,679,719	np
Burkino Faso	4,177,819	4,015,000	12,546,224	11,891,000	541,149	518,000
Cameroon	5,000,000	4,700,000	6,000,000	7,100,000	**1,000,000**	**1,370,000**
Canada1	2,972,038	11,289,000	**1,024,007**	**656,000**	10,216,083	10,172,000
Chile	3,460,530	3,404,000	5,823,126	5,401,000	1,071,950	1,251,000
Colombia	21,781,991	24,350,000	**2,765,173**	**3,514,000**	2,187,035	2,642,000
Comoros	43,200	47,000	131,200	139,000	na	na
Cote d Ivoire1,	195,459	1,145,000	2,240,667	2,069,000	396,750	372,000
Cuba	4,689,333	4,920,000	518,800	495,000	1,900,000	1,903,000
Cyprus	61,399	55,000	444,184	495,000	**566,107**	**278,000**
Denmark	2,189,804	2,222,000	**181,893**	**122,000**	10,455,402	9,767,000
Ecuador	4,690,000	4,516,000	1,894,000	1,810,000	2,423,000	2,327,000
El Salvador	1,495,000	1,243,000	20,000	20,000	317,000	308,000
Ethiopia	27,000,000	30,000,000	41,000,000	41,000,000	20,000	20,000
Finland	**1,648,000**	**1,315,000**	**83,000**	**61,000**	1,300,000	1,290,000
Grenada	4,000	4,000	**10,000**	**23,000**	**15,000**	**3,000**
Guinea	1,820,000	1,530,000	**1,400,000**	**965,000**	33,000	27,000
Hong Kong	**2,570**	**2,000**	487	na	**96,900**	**234,000**

Country	Bovines		Sheep + Goats		Swine	
	OIE	FAO	OIE	FAO	OIE	FAO
Hungary	**1,049,000**	**1,571,000**	**184,000**	**1,889,000**	**6,112,000**	**8,000,0**
India	199,670,000	193,328,000	155,920,000	160,957,000	10,640,000	10,450,C
Ireland	7.022.700	6.101.000	**8,695,000**	**5,873,000**	1,385,800	1,249,C
Israel	330,000	331,000	**350,000**	**490,000**	**40,000**	**100,0**
Israel CT	17,000	np	700,000	np	na	
Jamaica	**178,000**	**320,000**	**206,000**	**443,000**	**60,000**	**150,0**
Japan	5,024,000	4,873,000	**61,000**	**67,000**	10,783,000	11,335,C
Jordan	**76,318**	**40,000**	**3,824,230**	**2,450,000**	1,000	
Kenya	11.000.000	13.000.000	16.500.000	14.500.000	**300,000**	**105,0**
Korean Rep	2.813.815	2.269.000	**573,875**	**349,000**	5.927.504	5.046.C
Libya	**21,500**	**125,000**	6,754,000	6,700,000	na	
Madagascar	10,287,000	10,265,000	2,126,000	1,950,000	1,525,000	1,461,0
Malta	19.000	22.000	11,500	11,000	**70,000**	**102,0**
Mexico	28,675,000	31,460,000	15,538,000	15,532,000	16,502,000	15,786,0
Mongolia	2,730,456	2,849,000	19,886,234	20,209,000	**48,600**	**135,0**
Morocco	3,269,433	3,183,000	**23,155,059**	**17,869,000**	na	
Namibia	2,233,714	2,212,000	4,103,298	5,181,000	17,550	17,0
Netherlands	4,796,768	5,057,000	1,973,047	1,917,000	14,964,430	13,788,C
New Zealand	8,144,321	8,100,000	53,101,160	55,955,000	411,148	407,0
Niger	1,956,216	1,790,000	9,323,885	8,467,000	na	38,0
Norway	984,000	974,000	**1,148,000**	**2,301,000**	766,000	721,0
Oman	125,886	138,000	831,853	868,000	na	
Panama	1,399,487	1,399,000	**6,874**	**5,000**	256,361	256,0
Paraguay	**9,861,163**	**7,627,000**	496,582	459,000	**1,261,676**	**2,580,0**
Peru	3,968,000	4,042,000	13,900,000	13,973,000	2,416,000	2,417,0
Poland	**7,000,000**	**8,844,000**	**1,650,000**	**3,234,000**	20,000,000	21,868,0
Romania	**3,865,000**	**5,381,000**	12.997.000	15.067.000	11,396,000	12,003,0
St Kits & Nevis	**3,000**	**5,000**	**8,200**	**25,000**	**3,000**	**2,0**
Saudia Arabia	204,367	200,000	10,396,157	10,184,000	na	
Senegal	2,627,013	2,687,000	**3,297,610**	**6,631,000**	**159,827**	**300,0**
Sierra Leone	333,181	333,000	378.072	425.000	**800,000**	**50,0**
South Africa	**9,800,000**	**13,512,000**	**28,600,000**	**38,480,000**	1.200.000	1.490.0
Spain	4,975,500	5,063,000	27,452,000	27,597,000	18,260,400	17,247,0
Sri Lanka	1.699.000	1.477.000	540.000	480.000	75.000	84.0
Sudan	**36,331,000**	**21,028,000**	**53,879,000**	**35,977,000**	na	
Swaziland	607.513	740.000	**448,875**	**358,000**	na	28,0
Sweden	1,775,302	1,707,000	**183,544**	**419,000**	2,279,053	2,201,0
Switzerland	1,700,000	1,829,000	493,000	474,000	1,701,000	1,723,0
Thailand	**7, 268,604**	**5,631,000**	274,574	302,000	**8,552,954**	**4,859,0**
Tunisia	**380,000**	**631,000**	**4,360,000**	**7,603,000**	na	
United Arab Rep	**68,711**	**53,000**	**290,995**	**975,000**	na	
United Kingdom	11,721,496	11,641,000	**43,746,287**	**28,944,000**	7,626,103	7,520,0
USA	101,749,000	98,896,000	11.038.900	13.030.000	56,798,000	54,477,0
Vanuatu	150.000	130.000	12.300	11.000	55,000	60,0
Venezuela	14,622,152	13,368,000	**1,321,742**	**2,145,000**	2.956.133	2.445.0
Zimbabwe	5.375.889	6.374.000	2.734.371	3.123.000	271.575	305.0

Table 2. Relative agreement/disagreement levels in the reports to OIE and FAO

Livestock	10.0%-19.9%		over 20%		Totals (% c. disagreement)	
Cattle	14	(20%)	17	(24%)	31/71	(44%)
Sheep & Goats	9	(13%)	31	(44%)	40/70	(57%)
Swine	9	(15%)	17	(29%)	26/60	(43%)
TOTALS	32	(15.9%)	65	(32.3%)	97/201	(48.3%)

1. Underlined numbers are ≥ 10 - 19,9% different
2. na = not available though other livestock data were provided
3. Numbers in **bold** are ≥ 20% apart
4. OIE lists these two separate sountries individually, FAO lumps them. The OIE figures are brought together for comparison. FAO presents collective nu for "Czechoslovak", Malaysia, and the United Kingdom, while OIE presents data for the separate states and principalities.
5. np = not provided, ie FAO presented no livestock data for any species. Bulgaria is merely an example. Recently, these are characteristically newly eme countries.

Incidence of anthrax, and environmental detection of *Bacillus anthracis* in the UK

P.C.B. TURNBULL[1], JANE E. BOWEN[1], J.S. GILLGAN[2] and N.J. BARRETT[3]

[1]Anthrax Section, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, UK; [2]Notifiable Diseases Section, Ministry of Agriculture, Fisheries, and Food, Government Buildings, Tolworth, Surrey KT6 7NF, UK; [3]PHLS Communicable Disease Surveillance Centre, 61 Colindale Avenue, London NW9 5EQ, UK

Notifications of human cases of anthrax remain exceedingly rare while small but troublesome outbreaks continue to occur in livestock. The last confirmed human incident was a case of cutaneous anthrax in a casual labourer in London in August 1995[1]. The source of the infection was not established. The confirmed human case prior to that was a case of cutaneous anthrax in a worker in a woollen mill in Scotland in October 1991[4]. The case notified in 1992 remained unconfirmed.

Figures 1 and 2 and Table 1 are updated versions of those presented at the last International Workshop on Anthrax[8]. Perhaps most noteworthy among the livestock incidents was the outbreak in an intensive pig-rearing unit in 1989[2,9] which led to the study reported elsewhere in these proceedings[5].

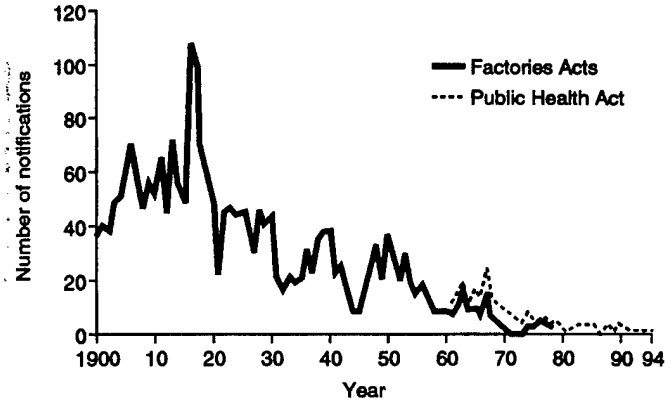

Figure 1. Human anthrax. Notifications under Factories Acts and Public Health Act United Kingdon 1900-1994

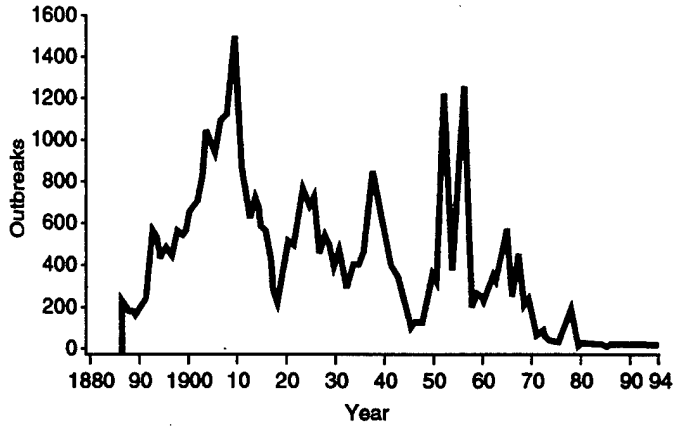

Figure 2. Reported cases of animal anthrax in Britain 1888-1994 (CVO reports [MAFF])

Although only 19 deaths were confirmed as cases of anthrax, the outbreak created husbandry problems which resulted in the ultimate slaughter and incineration of all 4492 animals on the farm.

The less anthrax is seen, the more emotive its occasional appearances become. Recent outbreaks in livestock have resulted in official obstacles to the disposal of the wastes reflecting concern which is out of proportion to the real hazards posed. Furthermore, as with the outbreak in pigs, interest from the media frequently raises public anxiety.

The finding of anthrax spores in environmental specimens similarly results in considerable public and official concern. Some perspective can be gained from Table 2, which summarises an analysis of 1342 environmental samples from 95 sites examined in the 8-year period, October 1987 to August 1995. In all, 43 (3.2 %) samples from 14 (15 %) sites yielded virulent *Bacillus anthracis*. Spore numbers exceeded an estimated 100 cfu/g in 13 of the positive samples, but 9 of these were from a single troublesome carcase burial site[3]. In the majority of samples, numbers were clearly very low and, in our view, represented no hazard to site workers taking sensible hygienic precautions. Risks for subsequent users of the site are also regarded as vanishingly small in these instances, but consideration is obviously given to either removal of the contamination or the most suitable subsequent use of such sites, for example, car parks. An attempt at providing guidance on assessing risks associated with environments known to be, or suspected of being contaminated with anthrax spores has been made elsewhere[6].

The isolation from environmental sites of avirulent *B. anthracis* that have lost one or both plasmids continues to be of interest since our formal report on this[7]. A dilemma exists over whether to report these to clients; while of no hazard in themselves, it is presumed they represent virulent parents and, as seen in Table 2, they are periodically isolated together with virulent counterparts. A molecular explanation of the point at which they lose their plasmid(s) in the germination, multiplication and sporulation cycle is looked forward to.

Table 1. Reported cases of human and animal anthrax in Britain 1989-95

Year	Human Notifications*	Animal		
		Incidents		Deaths
			Animals	Number†
1989	1	3	Cattle	3
		1	Pigs	19
1990	3	5	Cattle	6
1991	1	2	Cattle	2
1992	1	2	Cattle	2
1993	0	2	Cattle	5
1994	0	3	Cattle	5
1995	1	1	Cattle	1
		1	Ferrets	2

*No deaths reported
† Confirmed cases and not including animals which were treated and lived

Sources: Human: notifications and reports to OPCS, SHHD and HSE.
Animal: CVO reports (MAFF).

Table 2. Environmental specimens examined in the Anthrax Section, CAMR, during the period, October 1987 to August 1995†.

Type of site	Number of sites				Number of samples			
	Total	Number			Total	Number		
		V	AV	V + AV		V	AV	V+AV
Bone processing	17	1	1	3	228	6	8	2
soil					183	4	0	2
dust								
bone chips/charcoal					12	1	0	0
bonemeal					22	1	2	0
Tanneries	18	1	1	0	107	*2	0	0
Building materials	18	2	0	2	497	10	39	3
("horsehair plaster")								
Burial sites	13	1	0	1	133	*16	*2	0
Abattoirs	7	0	1	0	80	0	4	0
Laboratory/hospital	6	2	0	0	45	2	0	0
Sewage treatment works	5	1	1	0	193	2	2	0
Other *(fellmongers, Government Disinfection Station, manure etc)*	6	0	0	0	40	0	0	0
Not known	6	0	0	0	19	0	0	0
TOTALS	96	8	4	6	1342	38	55	5

†Modified from Table 1 in Turnbull (1996)[6]; reproduced by kind permission of the Editor and Publisher of *Land Contamination and Reclamation*
*From one site. V, virulent; A, avirulent (*cap*⁻)

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Anthrax in Russia

B. CHERKASSKIY

Central Research Institute of Epidemiology, 111123 Novogiveyersdaya str 3-a, Russia, Moscow

Anthrax has been a serious problem for veterinary and public health in the former USSR. Even in the beginning of the twentieth century on the territory of the former USSR about 40-60 thousand anthrax cases were registered in animals and 10-20 thousand cases in humans annually. The case fatality rate was 25%.

According to the official data, an average of 1200 cases of livestock anthrax were reported between 1971 and 1980 annually; the annual number of cases had fluctuated from 2750 cases in 1971 to 553 in 1980. An average of 440 cases of anthrax in human beings were registered annually; the annual number of cases had fluctuated from 658 in 1971 to 292 in 1980. The level of morbidity per 100,000 in animals averaged 0.36 and, in humans 0.17.

During the following decade, the level of morbidity in animals as well as in humans decreased; between 1981 and 1990 an average of 453 cases of anthrax of livestock had been registered annually; the annual number of cases had fluctuated from 1177 in 1981 to 181 in 1985. An average of 230 cases of anthrax in humans had been observed annually from 268 in 1983 to 176 in 1989. The average levels of morbidity 100,000 were 0.16 in animals and 0.8 in humans.

The level of anthrax morbidity was usually highest Kazakhstan and the republics of Central Asia. For exampl 1986-1990, about 50% of all registered cases in the for USSR were in Tadjikistan and 12% in Kazakhstan. The t number of cases just in Kazakhstan, Tadjikistan, Uzbekis Turkmenistan and Aserbaijan constituted 85% of all case the USSR. Russia accounted for only 11% of all cases, 50% which were registered in the territory of North Caucasus.

During these years, the incidence rate per 100, population in Tadjikistan was 17- fold higher than in the US on average, in Turkmenistan, 5-fold and in Kazakhstan, 3- 4- fold.

According to the official data an average of 94 case anthrax in humans were registered annually in Russia betw 1971 and 1980. The annual number of cases had fluctu from a peak of 151 in 1972 to a minimum of 46 in 1973. level of morbidity per 100,000 population was 0.06 an ave and fluctuated from 0.11 to 0.04.

During the following decade, the number of cases evidently decreased between 1981 and 1990 to an average of 48 cases of anthrax in human annually. The number of cases fluctuated from 72 in 1983 to 16 in 1989. The level of morbidity per 100,000 population was 0.03 on average and fluctuated from 0.01 to 0.05.

The main, recent characteristics of anthrax in Russia are:

1. Sporadic level of morbidity of animals and humans with rare occurrence of outbreaks and no tendency for it to decrease.
2. Disproportional territorial distribution of anthrax with predominance in the territory of North Caucasus.
3. The large proportion of human cases, about 95%, are observed in rural areas.
4. Anthrax peaks in animals as well as in humans are mainly reported in summer-autumn.
5. About 85% of anthrax cases in humans are connected with the infection in cattle.
6. A high association of products of animal origin as vehicles of transmission to people.
7. The disease is distributed mainly among stock-breeders and members of their families.
8. Prevalence of morbidity in adult males of ages 20-60 years, who take part in the slaughtering of infected livestock and in handling products of animal origin.

The main reasons for the recent epidemiological peculiarities of anthrax in Russia are:

1. Large numbers of endemic anthrax areas.
2. Insufficient levels of immunization of livestock against anthrax in endemic areas.
3. Delayed diagnosis of anthrax in animals.
4. The slaughter of infected livestock without preliminary veterinary examination of animals.
5. The distribution of products of animal origin among the human population without strong preliminary veterinary control.
6. Insufficient health education of the population as to the dangers of anthrax and the measures for prevention.

For example, in 1992, there was an outbreak of anthrax in the Kabardino-Balkarian Republic (North Caucases) . Twenty one men were infected and one girl of sixteen died. The history of this outbreak is typical: infected cattle had been slaughtered five minutes before death.

Anthrax is endemic throughout Russia. We collected the sufficient information on more than 10,000 endemic areas (settlements, villages, farms, pastures etc.) where plots of contaminated soil remain. Some of the collected anthrax foci were registered before 1990. The information includes data on geographical and administrative positions of the foci and the years when they were associated with anthrax activity (occurrence of cases in animals and/or humans).

In many registered anthrax foci, there have been no subsequent cases of anthrax in animals or humans. The authors believe that the *B. anthracis* in such foci have disappeared and the soil is no longer hazardous.

The result of the analyses of our data shows some of the anthracis foci associated with anthrax activity after 60-70 years of inactivity.

The geographical distribution of stable natural anthrax foci in Russia is almost limited to step and forest-step areas with a much lower number of foci occurring in forest, desert, semi-desert and other geographical areas.

The favourable conditions for the existence of stable anthrax foci are areas with predominantly fertile or brown soil. There is evidence of environmental multiplication of *B. anthracis* in soil with favourable pH, nutrient, humidity and temperature conditions. However the activity of the foci occur mostly in territories where there is well developed stock-breeding. Human anthrax is endemic in those agricultural regions of the country where anthrax in animals is common. Hence the reccurrence regularity of anthrax in the foci is different in different economical and geographical regions of Russia.

In 1929-1992, the proportion of anthrax foci was much higher in the southern part of Russia than in the northern and central parts. In the southern part the number of recurrent anthrax foci was also much higher. Moreover, in recent decades, 1973-1992, the recurrent foci were found only in the southern part of Russia.

For the most part, anthrax foci display activity only once, but in the southern part of Russia there are many foci displaying activity two, three or more times. In recent decades, the number of foci displaying anthrax activity more than twice appears to have decreased because of mass immunisation of animals.

The live spores of avirulent non-capsulated attenuated vaccine strain N 55 are used for immunisation of livestock in endemic areas. The livestock are revaccinated every year. In 1994, 87,718,000 animals were immunised: 47,469,000 cattle, 34,676,000 sheep and goats, 1,923,000 horses and 3,150 swine.

The similar vaccine from the avirulent non-capsulated vaccine strain "STI" for immunisation of humans in at risk occupations (veterinarians, workers who process hides, hair, bones and wool, agricultural workers who handle potentionally infected animals or contaminated products of animal origin, industrial raw materials in endemic regions regions) are used.

These people are revaccinated every year. In Russia every year about 130-150 thousand people are immunised against anthrax. For example 32,300 were vaccinated and 92,500 revaccinated in 1994.

Human anthrax in Turkey

M. DOGANAY

Department of Infectious Diseases, Faculty of Medicine, Erciyes University, 38039 Kayseri, Turkey

Anthrax is an endemic disease in Turkey[1]. A total of 10724 human anthrax cases were recorded between 1960 and 1969, 5377 human cases 1970, 1979, 4423 human cases 1980 and 1989, and 1779 human cases between 1990 and 1994. The annual numbers are shown in Figure 1. Although an average of about 561 human cases in a year were seen between 1970 and 1979, an average of 355 human cases were recorded for 1990 and 1994. The incidence of only human anthrax in Turkey is declining but is still far from being eradicated.

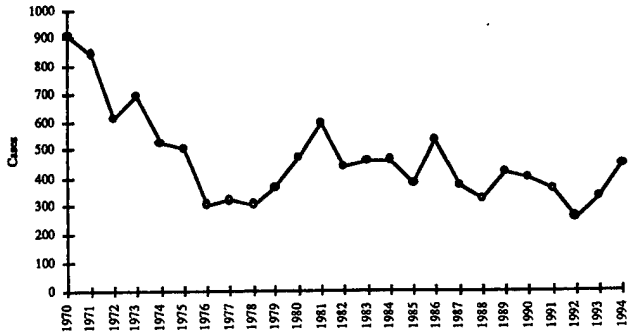

Figure 1. Incidence of human anthrax cases reported in Turkey 1970 - 94.

The majority of cases recorded were from the northeast, eastern and central part of Turkey. Some provinces (Kars, Van, Erzurum, Sivas, Corum and Konya) are high risk areas. Most patients were agricultural workers. The notified human anthrax cases were cutaneous. In our knowledge, few cases of gastrointestinal anthrax have been reported but inhalation anthrax has not been reported. It is believed that some cases of anthrax were not reported in Turkey.

Anthrax is a sporadic disease in Kayseri which is located in central Turkey. In the past 5 years (1990-1994), 23 cases of anthrax were recorded in this district by the Health Office. All cases came from rural areas. The source of infection was agricultural.

Mortality in humans is low. A total of 69 (0.02%) human cases died for anthrax between 1970 and 1994 in Turkey (Fig 2).

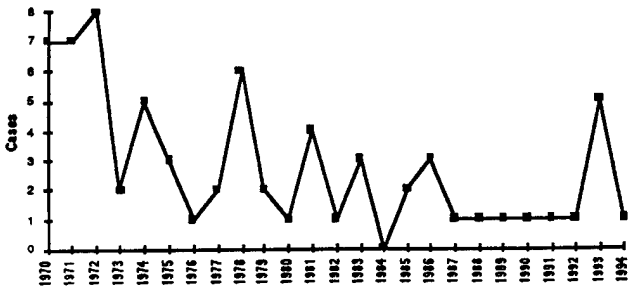

Figure 2. Numbers of fatal human anthrax cases 1970 -94.

Human or animal anthrax cases are reported to local public-health officials or veterinary service. All animals in the area involved are vaccinated against annually for three years and animal movements are also restricted. Disinfection of affected localities is carried out. Vaccination is free of charge. There is a national control programme and control measures are carried out by the Veterinary Department during outbreaks.

We conducted a seroepidemiological study in a leather processing center in Bor which is a village 150 km far from Kayseri. About 600 workers were employed in there. Of the 165 workers questioned, four workers gave a history of anthrax lesion 3, 7, 25 and 37 years ago respectively. Sera collected from 165 workers were tested for anti-PA and all samples were negative (serology was kindly performed by P. Turnbull, CAMR, UK and T. O'Brien, NMRI, USA).

Between September 1981 and July 1995, 113 cases of h anthrax were admitted to our in clinic. The source of infe was determined in 93 cases. In 89 cases, there was a histc handling of, or contact with infected dead animals or products; the animals involved were bovines or sheep. In 4 there was a history of consumption of meat from the carcas diseased cow or sheep. The source of infection in the rem 20 cases could not be determined but they were clea agricultural origin. Industrial anthrax was not observed [2,3].

Anthrax occurs throughout the year in this region bu majority of cases occur in the late summer and autumn. period of 1981-1995, accumulated case numbers seen i months of August, September, October and November 12, 26, 34 and 15 respectively. In this region, late summe early autumn are the driest season.

Of the 113 cases, 107 were cutaneous anthrax and 6 pr throat anthrax [2,3]. The distribution of the lesions in the ca summarized in Table 1. Between April 1989 and 199 cases of human anthrax were diagnosed and treated i clinic. The features of 15 cases are shown in Table 2.

Table 1. Distribution of lesions in 113 cases with anthrax.

Site of lesion	No. of cases	No. of deat
Cutaneous	**107**	-
Hands and fingers	79	-
Wrist and arms	10	-
Eyelid and face	11	-
Neck	2	-
Foot and leg	5	-
Tonsil	**5**	**3**
Tongue	**1**	-

Table 2. An analysis of 15 cases of human anthrax.

Number of patients	15
Female/Male	6/9
Source of infection	
Slaughtered and skinned of cow	8
Slaughtered and skinned of sheep	3
Chopping of suspected meat	3
Unknown	1
Incubation period (range, day)	2-13
Site of lesion	
Hands and fingers	10
Wrist and arms	3
Eyelid	2
Single lesion	11
Two or more lesions	4
Demonstration of bacilli from lesion	10/15*
Positive culture	5/15*
Antibiotic therapy	
Penicillin	11
Erythromycin	2
No therapy	2**

* Ten patients had received antibiotic before sampling

** These two patients were already on a course of therapy when first our clinic; this was completed.

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Incidence of anthrax in Indonesia, 1986-1995

S. HARDJOUTOMO

Balai Penelitian Veteriner, Bogor, Republic of Indonesia

For the 5-year period 1991-1995, the Directorate of Animal Health, Jakarta, reports that anthrax is still endemic in two provinces in Eastern Indonesia, West Nusatenggara (confined only to Sumbawa island) and East Nusatenggara (confined to a few islands. Control measures must be continued to minimize outbreaks and possibly to eradicate the disease[1].

Factors contributing to the decline of anthrax in animals in Indonesia during the past 10 years include: 1) the annual use of Sterne vaccine in endemic areas; 2) prompt diagnosis followed by regulatory activities to control outbreaks; 3) the use of antibiotics, especially penicillin, as early as possible in treating sick animals in the field.

Anthrax in animals has been known to occur in Indonesia since 1885[3]. Over the 10-year period 1976-1985, nine of 27 provinces of the country have reported anthrax, involving 4,310 animals[2]. In an infected province, usually two to three districts record anthrax cases or the disease is confined to only one island of the province. Cattle and buffaloes are the species most frequently affected by anthrax. Occasionally people contract the disease through contact with infected animals or consumption of contaminated meat. In Indonesia, anthrax is a notifiable animal disease. Strengthening the quarantine system, control of livestock movement, and vaccination of livestock in infected districts have to be carried out as control measures against anthrax outbreaks.

Over the 10-year period 1986-1995, a total of 1,880 cases of anthrax in animals were reported. This represents a 50% decline in anthrax incidence compared with the previous ten years (Table 1). Cattle and buffaloes were the species most frequently affected.

It was also evident that anthrax affected provinces declined dramatically (Table 2). In the 5-year period 1991-1995, only two provinces in the country, West Nusatenggara and East Nusantenggara, reported anthrax compared with five provinces in the previous 5-year period 1986-1990, and nine provinces for the 10-year period before that (1976-1985). Surprisingly, two other provinces that did not report anthrax in 1976-1985, Irian Jaya and Central Jawa, did experience outbreaks in 1986 and 1990 respectively. These responded to control measures (largely vaccination) (Table 2).

In some anthrax endemic districts, humans occasionally contracted the disease, but precise numbers of cases are difficult to obtain. Mortalities were few due to prompt diagnosis and antibiotic treatment.

Problems encountered in Indonesia include:

1) controlling the persistent outbreaks of anthrax in endemic areas;
2) providing rapid laboratory confirmation of field diagnoses of anthrax outbreaks; and
3) evaluating the effectiveness of the vaccination programmes conducted in endemic areas.

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Table 1. Anthrax in Indonesia. Number of animal deaths reported during 1986-1995 compared with 1976-1985.

The 10-year Period	Total number animals died	Order of species affected*
1976-1985	4,310	Cattle and Buffaloes Horses Sheep and Goats Pigs
1986-1995	1,880	Cattle and Buffaloes Sheep and Goats Pigs Horses

Source: Directorate of Animal Health, Jakarta
* Species in order of case frequency

Table 2. Provinces reporting anthrax in the periods 1976-1985, 1986-1990 and 1991-1995.

Provinces	Anthrax incidences during		
	1976-1985	1986-1990	1991-1995
Riau	+	-	-
West Sumatra	+	+	-
Jambi	+	-	-
Jakarta Raya	+	-	-
West Java	+	+	-
Central Java	-	+	-
South Sulawesi	+	-	-
Southeast Sulawesi	+	-	-
East Nusantenggara	+	-	+
West Nusatenggara	+	+	+
Irian Jaya	-	+	-

Source: Directorate of Animal Health, Jakarta

Experience with anthrax control in areas of Tanzania

S. F. H. JIWA

Dept of Microbiology and Parasitology, faculty of Veterinary Medicine, Sokoine University of Agriculture (SUA), P.O. Box 3 Morogoro, Tanzania

Summary and introduction

Anthrax has been sporadic in Tanzania in susceptible game, domestic food animals and pets for many decades. However records of original outbreaks are not available. Over the period 1964-94 the problems of inadequate hygiene measures and inability to control anthrax in game parks have posed the biggest threats to humans and susceptible livestock. Furthermore, a breakdown in extension services seems to be causing a significant slump in vaccination coverage. Locally produced Sterne strain vaccine is available only irregularly.

Materials and methods

Area

The three zones, viz Lake, Central and Eastern, covered in this report contain approximately 40% of Tanzania's total livestock population of 13.5 million cattle, 3.6 million ovines and 9.2 million caprines (Fig 1)[1]. These areas also include parts of Serengeti, Ruaha, Mikumi, Itenda and Selous games sancturies.

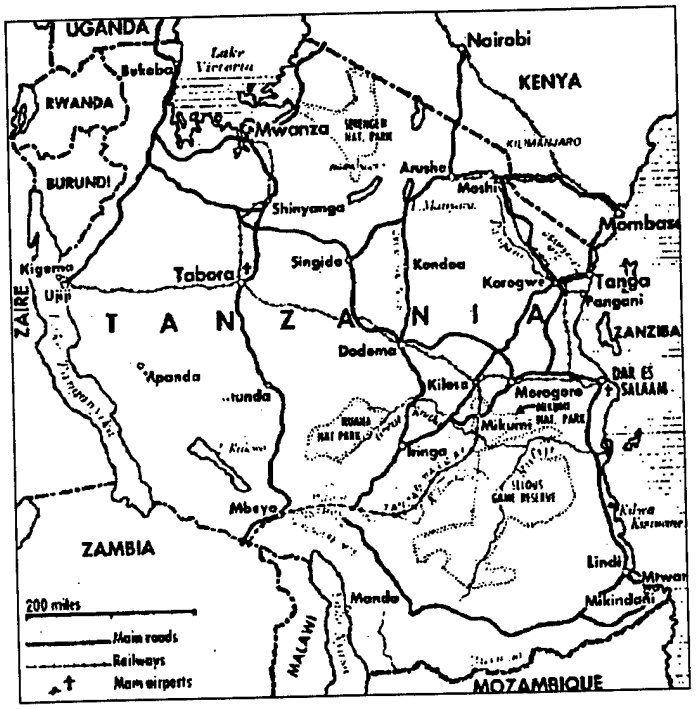

Fig. 1 Map of Tanzania: Administrative centres and game sanctuaries.

Diagnosis

Post-mortem of obvious cases is illegal. Ear-vein blood-smears were air dried, heat-fixed and Gram stained at district level and Giemsa stained at village level. Non-government organisation (NGO) establishments, like mission health centres, leprasoriums and teaching facilities, were utilised in the absence of governmental facilities.

Carcass disposal

In many cases, carcases are buried two meters deep in li in a minority of cases this is not done as the regu prescribe and the resulting shallow graves are opened hyenas and wild dogs. The carcasses are sprayed wit wash. Graves when two-thirds filled, are covered with *A seyal* var. *fistula* twigs and topped up with sand mixe whistling spikes of the same tree as a deterrent to scav animals[4].

Anthrax awareness campaigns

Veterinarians, physicians, school teachers, local elders organisation staff and government leaders are invol boost anthrax awareness among livestock owners, esp during large scale Rinderpest and DBPP vaccination exe

Wildlife outbreaks

Mikumi park recorded intermittent outbreaks, mainly and immediately after rainy periods. The principal involved even impalas, zebras and a variety of g Similar outbreaks in Tarangire, Mkomasi, Ruaha and parks reported anthrax in wild dogs which spread to d dogs[2]. In Singida region, an unconfirmed case of anthr elephant was reported near Elton's grave, Intend reserve, and in a hyena with two pups.

Wildlife vaccination

Saa Nane Island Game Sanctuary off the Lake Victoria Mwanza holds approximately 200 herbivores mainly bu elands, wildebeests, bush and water bucks, giraffes, impalas, gazelles and dik-diks. The sanctuary also h gorilla, two caged lions and two cheetahs all of whi vaccinated using blanthrax (combined black leg/ vaccine (Haskovo Public Health Labs Bulgaria), a Non-herbivores were sedated first with pentabarbitone tablets in baited meat. Artificially reared wildgame at that is, four lions, two cheetahs, two leopards, two zeb giraffes, two buffaloes and two elands were s vaccinated. The Singida animals are displayed regu various centres of Tanzania on important public hol part of wildlife awareness campaigns in urban areas[3].

Results and discussion

Nationwide vaccination as an effective control mea declined drastically in the recent years compared to before (Tables 1, 2). In the field, sporadic outbreaks to occur, predominantly in cattle, but also in sheep ar In game parks, anthrax remains largely uncontrolle anthrax poses an intractable problem when it is consid game areas constitute 25% of the area of Tanzania in million kms. Anthrax has continued to be endemic in Mkomasi, Ruaha and Selous game parks and these permanent potential source for further spread among and also domestic life.[2]

Table 1: Anthrax incidence in mainland Tanzania

Year	Deaths			Vaccination			Totals
		BOV	CAP	OVI	BOV	CAP	OVI
1992	916	024	7	238,142	112	2,718	240,972
1993	167	122	2	99,958	20,882	305	121,145
1994*	225	013	4	30,794	9,841	362	40,997
Incidents*	178	013	4	-	-	-	-

Legend: BOV = Bovine, CAP = Caprine and OVI = Ovine

Table 2: Anthrax incidence in Singida Region

Year	Incident	Deaths	Vaccinations*			Totals
			BOV	CAP	OVI	
1966	139	139	79,275	7,850	8,325	95,450
1967	105	105	76,724	8,732	4,755	90,211
1968	67	98	68,364	3,092	5,620	77,076
1969	131	150	59,108	5,694	3,240	68,042

*Blanthrax, Bactifabrin, Anthrax

Failure to implement hygienic regulations has helped to spread anthrax to man via contact with infected meat and consumption. Efforts need to be channelled to find solutions to these problems. Hyenas opening shallow graves were deterred in many cases by blending grave sand with twigs and thorns of *Acacia seyal* plant, which grows commonly in Tanzania[6]. Extension services need to increase anthrax awareness among rural populations and to include veterinarians, physicians, livestock owners, school teachers, village elders, and administrators. But all this can only succeed if a regular supply of adequately priced vaccine is made available to stock-owners.

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Anthrax in Western Province, Zambia

G.BBALO

Department of Veterinary and Tsetse Control Services, PO Box 910248, Mongu, Western Province, Zambia

Introduction

Anthrax, a disease of warm blooded animals caused by a Gram positive bacterium *Bacillus anthracis*, has been in the Western Province for more than 25 years. Isolated outbreaks were reported from 1970 to 1975 in Mongu and Senanga districts. In the same districts cases were recorded in 1981 and 1987. Thereafter a major epizootic occurred in 1990 to 1991 involving all districts, but at low incidence. No human cases were recorded in the 1970 - 87 outbreaks. In 1991, 184 humans were treated with 35 deaths, while in 1993 there were 209 treated cases with 34 deaths. It is difficult to give an exact number of cattle that have died from anthrax due to non-specific reports.

Epidemiology and spread of the disease

The reduction in disease prevalence up to 1975 was achieved by good vaccination coverage. Vaccinations virtually ceased from 1976 to 1989 and the disease was 'forgotten' as no cases were being reported . The cessation of vaccination for a period more than 10 years could have created a susceptible population while spores remained in the soils. Most of the outbreaks that occurred, except for extensions, were in the Zambezi flood plains which gets flooded for a period of 4 to 6 months from December to May.

Spread of the disease is associated with the socio-economic behaviour of the community in the Province. The transhumant system practised along the flood plains means that cattle change grazing areas each year. They use the flood plains from May/June to December and spend the remainder of the period in forest areas. With cattle being the hard currency of the community, changing hands, and hence places, is a common phenomenon. Cattle will change hands and move over distances through sales, payments for court cases and brideprice, gifts to relatives and friends, and short term loans for milking and manuring. Cattle products are also transported over distances indiscriminately. Under the short term load system, if death occurs while cattle are still in the custody of the keeper, the meat or hide will be retained or taken to the owner for proof of death.

Movements of cattle out of and into the Province are very strict, but less so between districts and not at all within districts. Cattle destined for slaughter outside the Province will be trekked on hoof to certain loading bays in nearby town centres. A number of deaths due to anthrax have occurred at these loading bays and soils tested at these centres have shown presence of anthrax spores. With the advent of drought in the Province, maize distribution centres have become death spots for oxen especially.

Reporting system

Western Province Veterinary Services, headed by a veterinarian, is divided into six districts which are also headed by veterinarians. Each district is subdivided into veterinary camps manned by a veterinary assistant (VA). The Province has a total of 77 veterinary camps. A camp has a population of cattle ranging from 2000 to 12000 and covers an area of 400 km^2 on average. A farmer suspecting a disease will report to the VA. A distance of 20 km may be walked. The VA must visit the kraal, take samples if necessary, and then travel to the district office, up to 100 km away, on foot or on bicycle. The district will then inform the provincial office of the suspected case(s). The office of the Director will then be informed. Samples collected from the animal will be forwarded to the regional laboratory for testing. Soil samples will be forwarded to the Central Veterinary Research Institute (CVRI) in Lusaka, a distance of about 500 km. The period it takes for the information to reach the district varies from a day to a month depending on the distance to the area and the reaction of the VA involved. Hence intervention may only come after a month.

Intervention/control measures

Anthrax is a notifiable disease in Zambia and when an outbreak occurs, the notifiable disease procedure is followed. Its control is currently financed with public funds.

Vaccination had been the main intervention measure used in the Province. Where coverage has recorded >80%, prevalence is reduced to isolated cases which will usually have been introduced after vaccination or have occurred because of missed vaccinations. In areas where few or no cases have occurred, vaccination coverage remains suboptimal. Other reasons leading to suboptimal coverage are;

- inadequate funds required to secure vaccines and to carry out vaccinations
- inaccessibility of some areas at vaccination times
- status of the vaccine at the time of inoculation

Diagnostic facilities and capability

A Regional Diagnostic Laboratory (RDL) is situated in Mongu. All specimens from districts are examined at the RDL with a part sent to the CVRI in Lusaka. No laboratory diagnosis is done at district level or, worse still, at the camp. The laboratory staff at RDL are adequately trained and therefore have the competence to carry out the tests.

Extension

Having experienced some difficulties in carrying out certain measures like the disposal of anthrax carcasses and vaccination with the communities, development of extension materials was embarked upon to educate communities on the disease and also educate staff, including non veterinary staff, on recognising the disease and how to handle the communities. It is hoped that through extension, communities will be active in preventing the spread to livestock and humans. The disposal methods available (Table 1.) can be used individually or in combination depending on the areas and number of carcasses. These are the only procedures for disposal of anthrax carcasses open to communities in Western Province.

Table 1. Options available in Western Province for disposing of anthrax carcasses

No.	Disposal	Requirements	Problems
1	Burning	One tonne of firewood	There is no firewood in flood plains Labour
2	Burying	Ditch 2m x 1.7m x 1.7m	Labour Dogs/jackals digging out
3	Leave to rot	Thorny shrubs	Dogs, jackals, birds and human interference

With improved extension services, it is hoped that fewer carcasses will be opened up, vaccination coverage will increase, reporting will be faster and livestock/products movements will be better controlled.

Problems encountered in the control anthrax

1. Lag period. It sometimes takes long periods to initiate control procedures because of poor communication.
2. Inadequate funds to secure required amounts of vaccine and other logistics for control.
3. Difficulties in restricting intra/inter district livestock and products movements.
4. Disposal of anthrax carcasses.
5. Community will, sometimes, wait until humans are affected before they report.
7. Lack of diagnostic facilities at district and camp levels.
8. Lack of facilities at camp level to allow for immediate reaction eg transport, simple microscope, telephones/radio and cooling facilities.

Evidence of anthrax in humans and animals in Nepal

[1]D.D. JOSHI, [2]A. PRADHAN and [3]N.P. GHIMIRE

[1]National Zoonoses & Food Hygiene Research Centre, Tachachal, P.O. Box 1885, Kathmandu, [2]Central Animal Disease Research Division, Nepal Agricultural Research Council, Tripureshwor, Kathmandu, and [3]Biological Products Division, Ministry of Agriculture, Veterinary Complex, Tripureshwor, Kathmandu, Nepal.

Summary

An epidemiological surveillance study, including a case study, was carried out in and around Kathmandu, Lalitpur and Bhaktapur districts which were affected with anthrax. Of 463 animals, 24 cattle, 2 pigs and 4 horses died from this disease as confirmed by blood smear examination. Imported animals and feed may have been the source of infection. The outbreak only ceased after vaccination of animals in the endemic areas with a live spore vaccine. Thirteen suspected human anthrax cases were admitted to Infectious Disease Hospital, Teku, Kathmandu. Future precautionary measures and recommendations for prevention and control of anthrax in Nepal are covered.

Introduction

Nepal has a predominately agricultural based economy and animal husbandry is an integral part of the farming system. The livestock sector is 15 percent of the national gross domestic product. Almost every household (5.6 persons on average) keeps an average of 5.6 head of cattle and buffalo as well as other livestock for food (milk, meat, eggs), manure, energy (draught, fuel, transport), wool, cash and barter income, financial security and religious purposes.

Livestock productivity is frequently threatened by bacterial, viral, parasitic, nutritional and other diseases, some of which are communicable to humans. Among the various bacterial zoonoses, anthrax, brucellosis, tuberculosis and salmonellosis are the most important diseases in Nepal. The signs and symptoms observed in both cattle and pigs suffering from anthrax were high fever, staggering, cold ears, throat swelling and death in 24 h[3].

Anthrax has been a public health problem in Nepal for the last five years. About thirty head of cattle died in a recent outbreak in Kathmandu Valley[2]. Similar outbreaks were recorded in Kavrepalanchowk, Kaski and Chitwan districts during the same period. The live anthrax spore vaccine manufactured by Biological Products Division (BPD) of the Nepal government was used for immunization[1]

Clinical cases of anthrax in thirteen humans, both cutaneous and pneumonic forms, have been diagnosed in the Kathmandu Valley by the Epidemiology Division. Samples were sent to reference laboratories abroad for confirmation by the Epidemiological Division of the Ministry of Health. The disease is a serious and increasing occupational problem in animal product based industries such as those dealing with wool, carpet, blood, bone and hides, which are now flourishing in the urban areas of the country but so far, little effort has been made for its prevention and control. From both economic and public health standpoints, a detailed epidemiological study to formulate effective control measures has been felt to be essential.

Materials and methods

An epidemiological team was sent to the endemic area for surveillance work. Data were gathered, processed and analysed. A technical team along with research officers went to conduct post mortems and collect blood smears. Samples were tested in the CADRD laboratory. A human case study was also carried out in Infectious Disease Hospital, Teku, supported by the Epidemiological Division, Ministry of Health, Teku, Kathmandu.

Results and recommendations

Animal case study

An outbreak of anthrax was first reported on December 5, 1991, in Bhaktapur district. A subsequent outbreak during January 1992 was reported in and around Kathmandu Valley area involving 24 cattle, 2 pigs and 4 horses. The epidemiological information is presented in Tables 1 and 2.

Postmortems were performed with strict precautionary measures. Dark bloody discharges from body orifices and hemorrhage of the mucous and serous membranes, lymph nodes and subcutaneous tissue were noted with splenomegaly, severe hemorrhagic enteritis and in some cases decomposition of the carcasses. The carcasses and other materials were then buried properly.

Table 1. Reported cases of anthrax during 1991/92[3]

Month	District	Place	Animal spp.	# of Animals	# of Deaths
Dec	Bhaktapur	Dadhikot	Cattle	300	4
Jan	Kathmandu	Kalanki	Cattle	9	4
Jan	Kathmandu	Chabhill	Cattle	12	1
Feb	Kathmandu	Maharaganj	Cattle	18	3
Feb	Kathmandu	Manditar	Cattle	12	2
April	Kathmandu	Baluwater	Cattle	4	2
April	Kathmandu	Kamalpokhari	Cattle	20	2
June	Kathmandu	Sinamangal	Cattle	4	2
July	Kathmandu	Royal Palace	Cattle	55	1
August	Kathmandu	Hathigainda	Cattle	4	1
August	Kathmandu	Balaju	Pig	15	2
August	Lalitpur	Nakhu	Horse	4	4
August	Lalitpur	Nakhu	Cattle	6	2

Table 2. Epidemiological findings of anthrax in Kathmundu Valley[3]

Location	# of Animals	# of animals affected	# of deaths	CFR (%)
Bhaktapur	300	4	4	100
Kathmandu	153	20	20	100
Lalitpur	10	6	6	100
Total in valley	463	30	30	100

Altogether 15 smears of blood, tissue, and fluid samples were positive by microscopy. Biological testing in guinea pigs and rabbits was positive for anthrax. Tests were done at the Central Animal Disease Research Division, Nepal Agricultural Research Council (NARC), Tripureshwor, Kathmandu, Nepal.

A commercial feed company had supplied bone meal in the outbreak area. Areas with no infection did not receive commercially prepared bone meal. Laboratory diagnosis has confirmed the involvement of the bone meal as the source of the outbreak.

Live anthrax vaccine was produced by the Biological Products Division (BPD), Veterinary Complex, Tripureshwor, Kathmandu was used in all districts, especially the endemic areas. After vaccination two cattle died of anthrax. It is possible that they were already incubating anthrax at the time of vaccination.

Human case study

Four suspected cases of anthrax in humans with enteric symptoms were admitted to the Infectious Disease Hospital in Teku, Kathmandu. These cases reported by the Epidemiology Division, Ministry of Health, Teku. No laboratory studies were done.
There were 9 suspected cutaneous anthrax cases among wool carpet industry workers with scabies-like lesions on their hands and fingers. However, no laboratory studies were done.

Recommendations

As a result of the outbreaks, recommendations were put forward for procedures to be adopted in the event of anthrax outbreaks in the future. These were broadly in line with WHO recommendations[4], emphasising correct sample collection and handling and disposal of carcasses, vaccination of associated animals and herds, appropriate surveillance and information gathering, collaboration between and training of livestock and human health officials, laboratory testing, and public awareness campaigns.

Acknowledgements

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4. World Health Organization. Guidelines for the surveillance and control of anthrax in humans and animals. *WHO/Zoon./93.170* 1993: 69-73.Table 1. Reported cases of anthrax during 1991/923

Anthrax - a continuing problem in southern India

M.K. LALITHA[1], DILIP MATHAI[2], KURIEN THOMAS[3], A.KUMAR[1], ALKA GANESH[4], MARY JACOB[5] and T. JACOB JOHN[1].

[1]Departments of Microbiology, [2]Medicine Unit I, [3]Medicine Unit II, [4]Medicine Unit III, [5]Dermatology, Christian Medical College & Hospital, Vellore, India.

Introduction

In India, a few sporadic cases and outbreaks of human anthrax have been reported earlier[4-11]. The North Arcot Ambedkar (NAA) district in the state of Tamil Nadu lies in the belt where the reported incidence of anthrax is the highest in India[12-16]. The first documented case of human anthrax in a patient with meningo-encephalitis (AME) in this region was noted in 1977[12]. At our institution after the first reported occurrence in 1977, until July 1995, we have seen 37 cases including an outbreak of AME in 1990[15]. The epidemiological features, the clincial presentation, laboratory diagnosis, treatment and preventive aspects of this life threatening illness seen in this region, are described in the report.

Epidemiological features in humans

The first documented case of human anthrax from North Arcot District was reported in 1977. Since then, the year-wise presentation of 37 cases of microbiologically proven anthrax is shown in Figure 1.

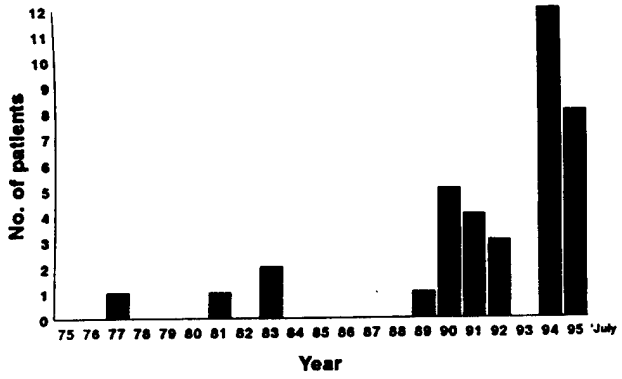

Figure 1. Anthrax cases at Vellore over 2 decades.

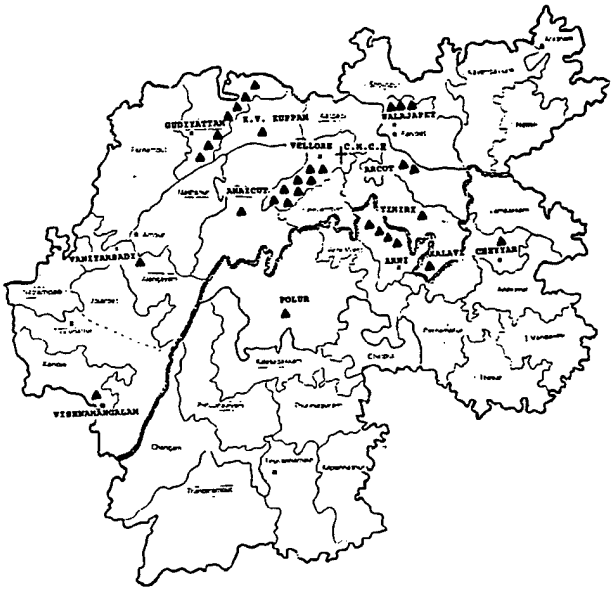

Figure 2: 'Spot map' of NAA District showing distribution of anthrax cases

The steep rise in the cases seen during the year 1994 and until June 1995 is of concern. The first outbreak of anthrax was reported from our centre in 1990. The epidemic occurred in the neighbouring state of Andra Pradesh, the region bordering Tamilnadu, in a group of villagers who were involved in handling the skin and meat of an infected sheep 15. Since then we have seen 2 outbreaks among family members; father and son in one and in the other, two young siblings, brother and sister. Most of the patients in this report came from NAA or nearby districts of Tamil Nadu. There were 8 cases from Vellore town itself. Figure 2 shows the geographical distribution of the reported cases.

There were 30 males and 7 females in our series. The age varied from 7 to 63 years. Most of the cases (27/37) were labourers and 25/37 gave history of handling animals or animal meat or hide. The other 12 denied history of contact with animals and, in 2 of these, cutaneous lesions were seen close to the eye with patients suggesting insect bites. This has to be considered in the light of previous reports from Africa of an insect vector in anthrax. It is noteworthy that no epidemic has been reported among tannery workers and in slaughter house pens.

Clinical presentation of anthrax

Meningo-encephalitis was seen in 25, cutaneous in 11 and septicemia in 1. One of the cutaneous cases presented as an 'injection abscess'. In eight cases of AME, cutaneous malignant pustules were noted. It is possible that in many instances, the cases are primary AME and the route of spread could have been through nasal mucosa. All the cases presented with headache, vomiting, high grade fever, change in mentation. In many cases, the onset was sudden with rapid deterioration in clincal condition of the patient. All patients had definite signs of meningeal irritation.

Anthrax in animals

There were two outbreaks of anthrax in 1994 among sheep in one pen, situated outside the hospital campus, maintained for collection of blood for microbiological work. Data obtained from regional Veterinary Centre, viz. Institute of Veterinary and Preventive Medicine (IVPM), Ranipet, revealed that there were several small outbreaks of anthrax among cattle, sheep and goats in this region (personal communication).

Primary cutaneous anthrax was seen in 11 cases. Seven of these had the classical black 'eschar'. A surrounding malignant oedema was seen in 10/11 cases. The lesions were seen on the arm in 5 patients, face and neck in 5 and involving both upper and lower limbs in one patient.

Diagnosis of anthrax

General laboratory findings

The cerebrospinal fluid (CSF) was 'haemorrhagic' in appearance in all but 2 cases. The CSF RBC count ranged from 100-50,000/cu mm and the WBC, 250-34,000/cu mm. The mean values of protein and sugar were 413 and 91 mgm%, respectively.

Microbiological features

The Gram stained smear examination of the CSF revealed large and thick Gram positive bacilli with truncated ends and the suggestion of 'capsule' around in all cases of AME. The organisms were arranged singly, in pairs or in short chains. The methylene blue stained smear showed long and thick bacilli, surrounded by an amorphous purplish area representing the capsular material. In cutaneous anthrax cases, the aspirated material showed the specific bacilli. However, the organisms were fewer in number. All 37 cases were smear positive which were confirmed by culture in 35 cases.

The organism grew readily on nutrient agar as large colonies with irregular edges revealing a 'ground glass' appearance of the surface. The colonies were completely non haemolytic on blood agar. The other pertinent features included absence of motility, slow liquefaction of gelatin and uniform susceptibility to penicillin. In a few instances, animal pathogenicity could be performed using guinea pig and the animals died of anthrax.

Treatment and outcome

All meningitic cases were treated with high 'meningitic' doses of intravenous penicillin, 2 million units every 2 hrs (12 to 24 million units/day) for 14 days. Patients with cutaneous anthrax who were seriously ill, were treated with intravenous penicillin 12-24 million units given daily in 6 divided doses. After 5 to 7 days, when the initial severity subsided and in those with less severe presentation, procaine penicillin 800,000 IU was given intramuscularly, twice daily. The total duration of therapy was 10 to 14 days. All patients with cutaneous anthrax responded favourably to treatment, while only two patients with AME survived. Most deaths occurred within 24 hours of admission. The cause of death has been attributed to intensive cerebral oedema.

Prevention and prophylaxis

Being an animal associated illness, the prevention largely depends on control of infection among livestock. The Sterne vaccine, which is a live attenuated spore vaccine, is manufactured in our district at the IVPM, Ranipet. Mass immunisation of the animal population is being resisted by the farmers for fear of the vaccine contributing towards the disease process. Legislation in meat handling do exist. However, it is difficult to change attitudes among the lower socio-economic villagers to discard the meat of dead animal or carcass. The data on the increased incidence of animal and human anthrax has been communicated to the local health care practitioners and public health workers through an existing health information 'net working' system.

Summary

The North Arcot Ambedkar District is one of the endemic areas for anthrax in India. An increase in the number of anthrax cases has been noted in veterinary and human practice in this area. Patients with meningo-encephalitis are increasingly seen in referal care centers like ours. Most cases occurred in labourers who gave a history of handling animal meat or skins of infected animals. The meningo-encephalitic form of the disease has a very bad prognosis. Twenty three of twenty five patients with this form of the anthrax died inspite of treatment with high dose penicillin. The typical bacilli are easily seen in the CSF in AME cases and are diagnostic of the condition. The cutaneous form of the illness has a benign course and responds favourably to pencillin treatment. In a few instances an 'insect bite' has been attributed as the cause of the cutaneous lesion. The disease needs to be prevented with proper legislation for meat handling as well as effective immunisation of animals.

Addendum

Since the preparation of this report, we have encountered nine more cases of anthrax in our region, three AME cases, five with cutaneous anthrax and one patient with only septicaemia with the clinical suspicion of meningitis. The local veterinary anthorities have been alerted about this problem once more and vaccination of animals has been stepped up.

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Anthrax surveillance and control in China

LIANG XUDONG, MA FENGGIN and LI AIFANG

Institute of Epidemiology and Microbiology, Chinese Academy of Preventative Medicine. P.O. Box 5, Changping, Beijing 1022 China

Summary

Human anthrax in China is largely a disease of agricultural workers and the main source of infection is the butchering and subsequent consumption of infected livestock. The disease is commonest among males during the summer and the cutaneous form accounted for 91.84% of cases reported. During the period 1985 - 1994 the average annual number of cases reported was 2115 and, following an outbreak in Tibet in 1989 in which 162 out of 507 cases were fatal, a body was set up in 1990 by the Ministry of Public Health headed by the Institute of Epidemiology and Microbiology to work on surveillance and control of the disease in the ten provinces where the incidence is high. The results of this surveillance are discussed and the vaccination programme described.

Introduction

Stations were established in the ten provinces where incidence of anthrax was high (Sichuan, Tibet, Inner Mongolia, Xingjiang, Qinghai, Gansu, Guangxi, Guizou, Yunnan and Hunan) to carry out monitoring on the environmental aspects, epidemiology, aetiology, serology and immunology of anthrax. Incidences of the disease were concentrated in a number of relatively low lying regions, especially after a dry spring followed by flooding during the summer. In China, there are vast areas with high levels of contamination, and when tested, 6.1% of environmental samples were positive for *B. anthracis* posing potential risks to human beings. The control of anthrax in China requires greater consideration, more resources and a long term programme.

As a result of a strict wool and hair quarantining system and improved working conditions in China, no cases of industrial anthrax have been reported for many years. Currently cases of human anthrax are generally confined to agricultural labourers and according to national statistical data, in the period 1985 - 1994 the annual average occurrence of anthrax was 2115 cases, with an incidence of 0.014 - 0.024 per million and a mortality of 2.35-12.97%. In 1989, during an outbreak of intestinal anthrax in the Changdu region of Tibet which lasted 6 months and spread to 34 villages in 5 counties, 162 died out of 507 human cases. This outbreak resulted in the formation in 1990 of a co-operative body working on anthrax surveillance and control. This body was founded by the Ministry of Public Health headed by the Institute of Epidemiology and Microbiology and included the surveillance teams from the ten provinces and autonomous regions. Support for this co-operative effort was supplied by other government organisations such as the Ministry of Agriculture.

General situation of anthrax in the period of 1990 - 1994

According to the national communicable disease report from 1990 - 1994, there were 9799 cases of anthrax registered in 21 provinces of China, of which 380 were fatal. The annual incidence was 0.0172 per million, with a mortality of 3.64%. As shown in Table 1, in the 10 provinces under surveillance, 72 outbreaks were reported with 8998 cases. Among th were 15 outbreaks, which were considered to be more se and are detailed in Table 2.

Table 1. Human Anthrax Cases in China 1990 -1993

Province	Number of cases	Incidence 1/100,000	Number of deaths	Percent mortal
Guihou	3188	2.384	130	4.07
Guangxi	1033	0.595	44	4.25
Yunnan	972	0.608	37	3.81
Hunan	332	0.133	12	3.61
Sichuan	335	0.076	44	13.1
Tibet	327	3.395	34	10.3
Xingjiang	1086	1.781	8	0.74
Qinghai	131	0.713	4	3.05
Gansu	215	0.232	3	1.39
Inner Mongolia	169	0.189	0	0
All of China	8122	0.195	324	3.99

Epidemiology

Sources of infection

The surveillance scheme provided insight into the n sources of infection in China, of which the most impo were shown to be cattle in the south and sheep in the n This reflected the numbers of the different species kept in areas, cattle predominating in the south west and sheep in north west. The numbers of different species infected in regions are shown in Table 3.

Transmission patterns

The major route for transmission of the disease to hu beings was direct contact with infected livestock and consumption of undercooked infected meat. Occasion transmission occurred through an insect vector, following b by bloodsucking arthropods. The mode of infection in human cases is shown in Table 4.

Epidemic patterns

Geographic effects

Incidences of anthrax were not restricted to a particular alti range, with occurrences from the Sichuan Basin at an alti of less than 100 m to the Qinghai Tibet Plateau at over 400 However, within the largely rural and pastoral areas affec incidences were concentrated in the lower-lying areas.

Seasonal effects

Episodes of anthrax occurred in all seasons in China but 82 of cases occurred between June and September after the spring and summer floods. The duration of the average an epidemic was approximately 150 days.

Table 2. Statistical data of fifteen severe anthrax outbreaks

Province	Date	Number of affected villages	Number of herds infected		Number of human cases	
			Infected	Died	Infected	Died
Liucheng Guangxi	6/91	10	55	55	44	2
Bama Guangxi	6/92	35	235	155	68	6
Donglan Guangxi	4/93	30	59	51	29	2
Changde Hunan	9/91	4	21	12	16	0
Lengshuijiang Hunan	10/92	4	26	14	16	2
Huaihua Hunan	9/93	6	29	17	16	0
Lonchuan Yunnan	7/91	4	35	32	18	0
Fengqing Yunnan	5/92	5	26	26	29	0
Langang Yunnan	8/93	1	49	19	72	1
Hongyuan Sichuan	7/91	1	43	43	20	4
Lodian Guizhou	4/91	3	71	34	16	2
Helin Inner Mongolia	6/91	1	54	51	32	0
Zepu Xingjiang	9/90	24	219	202	146	0
Wusu Xingjiang	6/90	6	110	80	34	1
Atushi Xingjiang	5/93	4	94	81	88	0
Totals		138	1126	872	644	20

Table 3. Livestock infected with anthrax in the 10 provinces under surveillance

Province	Total infected	Buffalo	Percentage	Goat	Percentage
In south west China					
Guizhou	338	207	61.2	41	12.1
Guangxi	421	334	79.3	37	8.8
Yunnan	723	460	63.6	118	16.3
Hunnan	386	253	65.5	31	8.1
Sichuan	267	221	82.7	15	5.6
Tibet	191	110	57.5	19	9.9
In north west China					
Xingjiang	628	120	19.1	411	65.4
Qinghai	346	101	29.1	152	43.9
Gansu	329	74	22.5	188	57.1
Inner Mongolia	486	78	16.1	298	61.3

Table 4. Source of infection in 593 cases of human anthrax

Source of infection	Number of cases	Percentage
Dismembering animals	287	48.39
Consumption of infected meat	192	32.37
Processing infected meat	97	16.35
Contact with patients	10	1.68
Environmental contamination	4	0.67
Others	3	0.5
Totals	593	100

Distribution of anthrax in the human population

Within a human population, susceptibility to anthrax is independent of age and sex. As an illustration of this, the oldest sufferer recorded during the outbreaks was 84 years and the youngest 3 months old. The majority of patients were however concentrated within the age range of 20 - 49. There was found to be a higher incidence rate in males than in females which is probably related to the increased chance of males coming into contact with the infectious sources, particularly livestock, in their employment. Traditional rituals performed by the inhabitants of certain regions could have resulted in extraordinarily high numbers of cases, as in the Hetian district in southern Xinjiang, where the high incidence in children (86.54 per million) was in part attributable to parents using animal oil to protect children's skin, and furs for bed making. Table 5 shows the statistical data associated with age distribution in 470 human cases.

Table 5. Age distribution of 470 anthrax patients

Age	Number of cases	Percentage of total
<1	34	7.2
1 - 10	36	7.6
11 - 20	130	27.6
21 - 30	97	20.6
31 - 40	82	17.4
41 - 50	56	11.9
>50	35	7.4
Totals	470	100

Occupational distribution

In China, other than in agriculture, no cases of industrial anthrax have been reported for many years and this is no doubt a reflection on the implementation of a strict wool and hair quarantining system and improved working conditions in industry. All the cases registered in south west and north west China occurred in rural and pastoral areas. Peasants and herdsman were mainly affected resulting from their contact with livestock. Table 6 shows the occupation of 1910 anthrax cases.

Table 6. Distribution of 1910 anthrax cases by occupation

Age	Number of Cases	Percentage of total
Peasant or herdsman	1557	81.5
Student	170	8.9
Child	54	2.8
Worker	43	2.3
Officer	39	2.1
Others	47	3.6
Totals	1910	100

Clinical symptoms

In China, the cutaneous form of anthrax was most common (91.84%), the intestinal less so (5.97%), and the pulmonary form was rare (2.18%). In more than 90% of the 1260 cases of

cutaneous anthrax, the primary site of infection was an open wound on an uncovered area.The symptoms of the disease were fever and enlargement of local lymph nodes. In the early stages of a cutaneous infection it is possible to effect a cure. However intestinal and pulmonary anthrax are frequently misdiagnosed and treatment is therefore delayed resulting in increased mortality. Table 7 shows the clinical symptoms of 194 cases.

Table 7. Clinical symptoms in 194 cases of anthrax

Symptom	Number of cases	Percentage of total
Temperature <38C	122	62.8
>38C	72	37.2
Characteristic skin lesion	154	79.4
Lymphadenitis	67	34.5
Pain	103	53.1
Abdomenal pain or diarrhoea	57	29.4
Nausea or vomiting	26	13.4
Serious oedema	4	2.1
Percussive pain in the kidney region	78	40.2
Anaemia or haemorrhage	84	43.3

Epidemic modes

The majority of the episodes of anthrax were sporadic, small outbreaks. Only on 4 occasions were more than 10 human cases involved, 14 episodes had 5 - 10 cases, 72 had 2 - 5 and in 48 episodes only 1 person showed signs of infection. More than 90% of the outbreaks were followed by at least one year, commonly 3-5 years, of freedom from infection in the area. Of 173 outbreaks, only in 16 instances did another outbreak occur in the same region the next year. The longest epidemic lasted 7 months and the shortest only 20 days.

Isolation of bacteria

In epidemic areas, 1455 environmental samples were collected and underwent analysis; 6.1% were positive for *B. anthracis* and 89 strains were isolated. Among the positive samples a large percentage, (18.1 %), were from soil. During the epidemic period, the percentage of positive samples taken from the soil surface (< 10 cm) was 17.23 %, and from depths greater than 10 cm (3.85 %). This however did not apply at other times when the position was reversed with the percentage of positive soil surface samples (3.23 %) being lower than those obtained from deeper layers (10.75 %, $p<0.01$). Of the water samples analysed, 2.14 % were positive for *B. anthracis* and strains were also recovered from the bodies of catfish and ducks living in a pond polluted by the carcasses of animals. *B. anthracis* was detected in 74.31% of the faecal specimens collected from suspected cases of intestinal anthrax. A number of avirulent strains of *B. anthracis* devoid of a capsule were also isolated from these specimens. Of the wool and leather samples tested, 22.30 % were positive. The percentage of these samples testing positive implied that the environmental contamination by *B. anthracis* is serious and persistent posing risks of further outbreaks. Determination of the plasmid spectrum of the strains isolated found that all of the strains harboured pX01 (110 MDal) and pX02 (60 MDal).

Serological surveillance

In the epidemic areas, 1688 sera samples were collected from the resident population. Anti-capsule antibody titres were determined and the anti-PA antibody titres determined by ELISA. In the areas with outbreaks, anti-capsule antibody was present in a higher proportion of samples (29.1%) than in other surveillance areas (7.52%) ($p<0.01$). In areas where outbreaks had occurred, the antibody dynamics in the resident popul were similar to those in sufferers. Antibodies appeared 1 v after the start of the outbreak, reached a peak at 1 month then declined to the levels prevalent in non-anthrax a within 8 months. It was therefore concluded that in epidemic areas, mild, atypical and subclinical infections be very common.

Vaccination

A co-operative system for the control of anthrax established in the provinces where epidemics occu According to the observed main sources of infection in different areas, the appropriate key livestock were targete the vaccination programme. Vaccination was performe spring (February to March) to ensure that immunity to ant could be built up prior to the epidemic season. A mas vaccination programme was carried out in the high risk a around the outbreak points. In the human population, samples of sera were collected 1 - 3 months post vaccin and the anti-PA titre determined to confirm the efficienc vaccination.

Environmental surveillance

One year after 14 episodes, 420 soil samples were colle and analysed. The persistence of contamination was confir by the isolation of 7 strains of *B. anthracis* (1.66 Persistence of the organism in the environment is a pote danger for humans and livestock. The inadvertent disturb of a corpse buried 64 years before led to the first human in an outbreak of anthrax in Hunan province in July 1 which spread to 11 counties. This shows that it is a necessit dispose of anthrax corpses properly to minimise the possib of persistent environmental contamination leading to fu outbreaks in the future.

Conclusion

The surveillance system, which had been established, fc that anthrax occurred mainly in the rural areas covering a expanse of south west and north west China and that there a considerable level of environmental contamination. The r sources of human infection in different parts of China v determined and the chief cause of infection was shown tc the butchering and consumption of infected animals. commonest human cases were found to be young m because of their greater exposure to infected livestock. spring months followed by flooding in the summer appeare induce outbreaks. Outbreaks tended not to occur in the s area in the following year and where they did occur there v subclinical infections in humans and animals.

In China, a surveillance system has been establishec monitor various aspects of anthrax, this still requ strengthening and legal measures need to be taken to impr the control measures. Control of the disease is a continuing arduous task. Propaganda and education need to be used stop the butchering and consumption of infected animals improve the reporting system, to cause the correct treatmer anthrax carcasses preferably by burning and to strengthen vaccination programme in order to control anthrax in future.

Acknowledgements

The authors would like to thank their colleagues in the 10 provinces participated wholeheartedly in the surveillance and to Professor Liu Bing and Professor Dong Shulin who read this paper and provided valu thoughts and opinions.

Serological detection of human anthrax in Korean outbreaks 1992-1995

HEE-BOK OH, KYUNG-SEOK PARK and KEE-DUK PARK

Department of Microbiology, National Institute of Health, 5 Nukbun-dong, Eunpyung-ku, 122-701, Seoul, Korea.

Summary

Three human anthrax outbreaks occurred in Korea from 1992 to 1995. They were clinically suspected as oropharyngeal or gastrointestinal anthrax, and were related to the consumption of contaminated raw meat from cattle. In an attempt to confirm human anthrax retrospectively, 19 patients out of 43 cases and 18 healthy control individuals were studied. Antibodies to protective antigen (PA) of *Bacillus anthracis* were detected using enzyme linked immunosorbent assay (ELISA) and western blots. By ELISA, 17/19 patients were positive and no control sera were positive. Anti-PA antibody could be confirmed in 17 patients using western blots. The results obtained indicated that all three recent outbreaks in Korea could be serologically confirmed as anthrax. The anthrax PA IgG capture ELISA detection system was highly sensitive and specific and proved to be useful for the detection of antibody responses during the course of *B. anthracis* infection.

Introduction

In the early 1900s, bovine anthrax was one of the most important bacterial zoonotic dieseases in Korea, with an average of 526 cases annually in the 36 year period from 1907 to 1942. From 1952 through 1968, 4 outbreaks with 85 human cases of anthrax were recorded in Korea. Most (92.9%) were intestinal anthrax and were related to the consumption of meats of anthrax-infected cattle[1]. From 1969 to 1991, following extensive efforts on prevention of bovine anthrax, there had been no record of human anthrax cases, and it was thought that Korea was anthrax free.

However, from 1992 to 1995, 3 outbreaks, with a total 43 of cases, were clinically suspected as anthrax, each of which related to the consumption of contaminated beef or bovine brain and liver[3,4,5]. While the outbreak which occurred in the Kyeongju district was bacteriologically culture proven, the other two outbreaks remained to be confirmed (Table 1).

In an attempt to confirm human anthrax retrospectively in the 3 outbreaks, we assayed available acute and convalescent paired sera from 19 of 43 cases for the detection of antibodies to protective antigen (PA) of *Bacillus anthracis* using enzyme linked immunosorbent assay (ELISA). Western blots were also performed for confirmatory demonstration of PA antibody in patient sera.

Materials and methods

Sera from 19 patients, collected in acute and convalescent phases, and 18 healthy individuals from the same village or occupation as the patients, were studied. *B. anthracis* cell associated antigens were extracted with sodium dodecyl sulfate[2] from the strain isolated from the patient K-3 in Kyeongju. Baculovirus expressed *B. anthracis* PA antigen, monoclonal antibody (Mab) against protective antigen (Mab 14B7-1-1) and standard positive (vaccinated) and negative control sera were kindly provided by Cynthia Rossi, USAMRIID. Antibody titers to anthrax PA were assayed by using anthrax PA IgG capture ELISA system using Mab 14B7-1-1 as a capture antibody. Titers of test sera were read as the last dilution producing an absorbance above the OD of the mean plus 3 standard deviations of 50 tests of negative control sera. The titers of acute and convalescent sera were also compared. For the western blots, cell extracts or PA antigen were electrophoresed on SDS polyacrylamide gels and transblotted and reacted with sera diluted 1:100.

Results and discussion

In the ELISA test, the OD cut off value of negative controls (in 1:100 dilution) was 0.123. In the Daechon incidents, 11 out of 13 cases showed seroconversion, mostly with linear increase of titers through the time course, from negative (in 1:100 dilution) to positive (ranging from 1:100 to 1:6400). But the sensitivity of the reaction could not be calculated accurately as the late convalescent sera were not available in two negative cases. In Kyeongju outbreak, 2 cases showed definite seroconversion with acute and convalescent sera, and the other 2 cases (K-3, K-4) showed only a low level of antibody responses (*B. anthracis* was isolated from patient K-3). In two cases from Seoul, we could confirm the seroconversion of early (1:3200) and late convalescent serum (1:102400) from one patient, and the other also showed a high titer (1:3200) of IgG in convalescent sera (Table 2). Negative or low level of antibody responses were interpretated as a result of antibiotics treatment of patients in early phase. The sera were also analysed by western blots using PA antigen, and we could confirm the presence of specific antibodies againt PA in patients of anthrax including two negative cases in ELISA tests (Figure 1). In most of patient sera, IgG and IgM antibodies against *B. anthracis* antigens were also

TABLE 1. Recent outbreaks of human anthrax in Korea (1992-1995)

Year	District	No. of outbreaks	No. of cases	No. of deaths	Clinical symptoms	Isolation of *B. anthracis*
1992 Oct.	Daichon	1	13	0	Oropharyngeal	Not isolated
1994 Feb.	Kyeongju	1	28	3	Gastrointestinal & Oropharyngeal	Isolated from patients, cow meat & soil
1995	Seoul	1	2	1	Gastrointestinal	Not isolated

Table 2 Antibody responses against Protective Antigen of *Bacillus anthracis* in oropharyngeal or gastrointestinal anthrax patients measured by IgG capture

Outbreaks in	Patient no.	Antibody titer					
		Day 3	Day 7	Day 10	Day 15	Day 17	Day 21
Daechon (Oct. 1992)	D1	<100	-	100	-	400	-
	D2	<100	-	800	-	1600	-
	D3	<100	-	<100	-	200	-
	D4	<100	-	800	-	NT	-
	D5	<100	-	400	-	800	-
	D6	<100	-	<100	-	NT	-
	D7	400	-	1600	-	1600	-
	D8	<100	-	<100	-	400	-
	D9	<100	-	<100	-	NT	-
	D10	<100	-	1600	-	6400	-
	D11	<100	-	1600	-	NT	-
	D12	<100	-	<100	-	1600	-
	D13	<100	-	100	-	6400	-
Neg (n=8)		-	-	<100	-	-	-
Kyeongju (Feb. 1994)	K1	<100	-	-	-	-	800
	K2	100	-	-	-	-	1600
	K3	<100	-	-	-	-	100
	K4	<100	-	-	-	-	100
Neg (n=4)		<100	-	-	-	-	-
Seoul (Feb. 1995)	S1	-	3200	-	102400	-	102400
	S2	-	-	-	3200	-	3200
Neg (n=6)						<100	

NT, Not Tested -, Serum not Available <100, Negative result at 1:100 dilution
Neg: Negative Control, number (n) of Negative Controls is indicated.

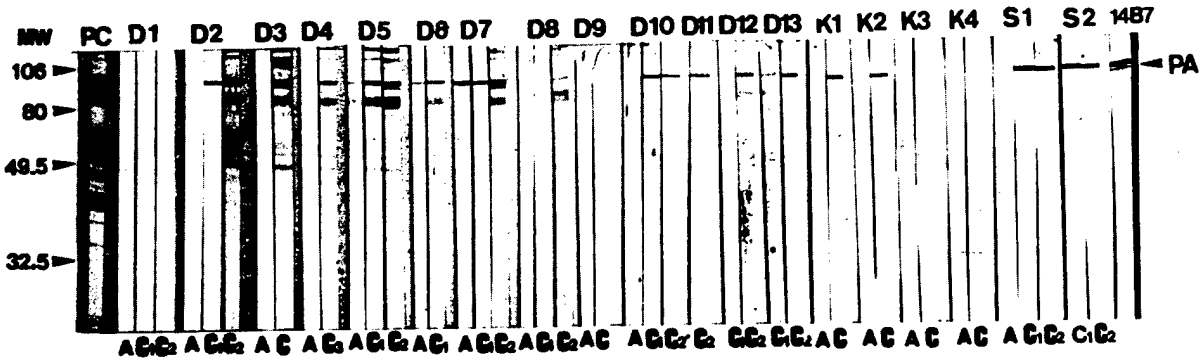

Figure 1. Detection of anti-PA antibody in sera of anthrax patients by using western blotting. Baculovirus-expressed PA was electrophoresed in 10 polyacrylamide gels and transblotted in Mini-Protean II (Bio-Rad) and reacted with sera diluted 1:100. Lane Pc: positive control sera (vaccinated). I correspond to numbers listed in Table 2. Strip 14B7 was stained with anti-PA monoclonal antibody. Molecular weights (kDa) of the standard proteins acute phase serum; C_1: early convalescent serum; C_2: late convalescent serum.

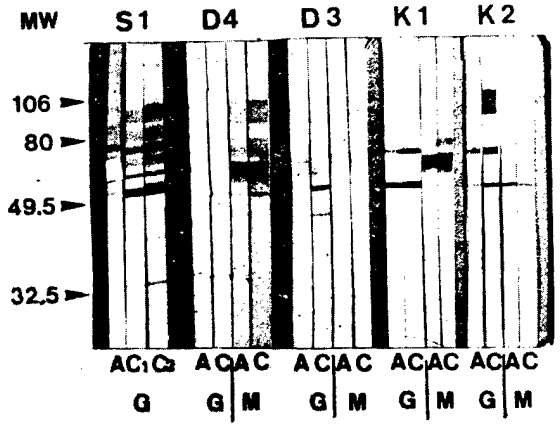

Figure 2. Western blot analysis of antibody responses of patients during the course of *Bacillus anthracis* infection using SDS-extracted cell-associated antigen. Cell-associated antigen was prepared with *B. anthracis* strain Kyeongju isolated from patient K-3. The numbers at the top of strips correspond to numbers listed in Table 2. Molecular weights (KDa) of the standard proteins used. A: acute serum; C_1 early convalescent serum. C_2: late convalescent serum; G: detection of IgG, M: detection of IgM.

detected in western blots using cell associated antigens (2). And no normal control serum showed any response PA antigen (data not shown).

In conclusion, all three recent outbreaks in Korea co serologically confirmed as anthrax, and *B. anthrac* detection systems using ELISA and western blottings a useful for the diagnosis of enteric anthrax patients.

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Incidence ou prévalence de la fièvre charbonneuse en Haiti - efforts de lutte

*J.N. PIERRE et J. VALBRUN

*Agr/D.M.V, Ministère de l'Agriculture, Damien, Port-au-Prince, Haiti *(Authors unable to attend the workshop)*

Les zoonoses majeures comme la Rage, la Tuberculose, la Fièvre Charbonneuse etc... continuent à faire beaucoup de victimes en Haiti. Cette dernière, qui depuis la période coloniale (il ya a environ 2 siècles), constituait avec le tétanos deux grands fléaux dont souffrait la population figure, parmi les plus redoutables en raison de ses conséquences économiques et hygiéniques, à l'heure actuelle.

Incidence

La fièvre charbonneuse due à *Bacillus anthracis* est enzootique à Haiti. Tous les neu départments géographiques sont affectés. Aucune étude n'a été menée pour déterminer les conséquences économiques et hygiéniques de cette maladie à l'échelle nationale mais les données partielles resceillies reflètent l'importance de cette zoonose (voir tableau suivant):

Tableau 1: Quelques données partielles sur l'incidence de la fièvre charbonneuse en Haiti

Departement	Cas humains	Cas animaux	Periode
Sud	100		Moyenne 1an
Nord	700*		Déc. 93-nov 94
Nord-Est		43	13 mai-26 juin 95
Artibonite		8	Non spécifiée
Grand-Anse	2		Janvier 1995

*Données enregistrées dans un seul hôpital

Il fait savoir que les cas animaux sont rarement déclarés. Beaucoup de cas humains ne sont pas non plus répertoirés pour être enregistrés. Leur nombre peut être estimé à plus de 2,000 par an en moyenne pour le cinq dernières années. Pour chaque humain déclaré à la Direction de la santé publique, il y a au moins un autre qui ne l'est pas, en raison de défaillance administrative.

Efforts de lutte

Pendant longtemps et jusqu'à nos jours, les interventions pour la lutte contre la fièvre Charbonneuse ont toujours été de type 'Pompier'. Aucun programme de lutte à long terme n'a été entrepris. Pour une compagne de vaccination donnée on n'est jamais parvenu à protéger 25% des espèces animales sensibles, faute de moyens et de motivatiob. Le tableau suivant donne une idée du statut immunitaire de ces dernières abandonnées dans des zones maudites par *Bacillus anthracis*:

Tableau 2: Nombre d'animaux (ruminants et equins) vaccinés par année à l'échelle nationale

Année	Nombre d' animeaux vaccinés
1992	248807
1983	212956
1984	--
1985	993103
1986	--
1987	--
1988	1491
1989	--
1990	--
1991	287000
1992	--
1993	16738
1994	114389

Commentaires et conclusion

L'ignorance de la population et l'absence de mesure de quarantaine interne favorise trés largement l'extension de la maladie. Le Nord et le Nord-Est où la fièvre Charbonneuse était pratiquement méconnue connaissent maintenant les plus fortes incidences. Au fil du temps, les efforts de prophylaxie médicale méritent d'être de plus en plus soutenus et les dépenses pour combattre la maladie deviennent de plus en plus importantes.

Dermal anthrax, probably originating from a XVth century goat leather "cordovan" and apparently attributable to a non-capsulating *Bacillus anthracis*

M. PRZYBYSZEWSKA[1], J. MATRAS[2], M. GÓRECKA[1], J. KNAP[1], M. BARTOSZCZE[2] and J. MIERZEJEWSK[2]

[1]Epidemiological Service, Ministry of Internal Affairs, Warsaw, Poland. [2]Military Institute of Hygiene and Epidemiology, Pulaway

A 37-year old male conservator of the National Museum, employed to preserve goat furniture covering leathers (cordovans) dating from a XVth century castle in Pieskowa Skala, Poland, and suffering from a pre-existing B-cell plasmocytoidal lymphoma with low malignancy, was admitted to hospital because of the appearance of blisters filled with brown haemorrhagic fluid on the palm and on forearms. The blisters were not painful. Concomitant oedema and lymphatic nodes vessels` reaction was present.

The patient mentioned that the first blister had appeared just after he had pricked his palm with a nail while extracting it from leather furniture covering. Leucocytosis was 19 200, thrombocytes 125 000/mm³. No Herpes virus was found in the fluid taken from the blister contents. Large numbers of Gram-positive rods in chains, without capsules, were found on microscopic examination of the blister contents.

Growth of grey, smooth colonies, with no haemolysis was obtained on solid media incubated aerobically and anaerobically. When 0.5 ml of broth culture supernatant was administered intraperitoneally to three mice, all survived.

The isolate was designated as *Bacillus cereus* by means of API 50 CHB tests (*B. pseudoanthracis, B. cereus* var. *anthracis* according to older nomenclature). An Ascoli reaction was positive. The isolate was susceptible to the following antibiotics: gentamycin, amikacin, vibramycin, amoxycillin with clavulanic acid, erythromycin and clindamycin, and partially suscepible to penicillin, while resistant to cefuroxim, lincomycin and cephradine.

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Editor's note

This is the third clinical case we are aware of associated with "capsule negative" isolate of *Bacillus anthracis*. Dr Liang Xudong and colleagues, in their paper earlier in this volume on anthrax surveillance and control in China, record isolation of non-capsulating *B. anthracis* from faeces in association with suspected intestinal anthrax. We ourselves received, for confirmation of identity, a blood culture isolate made on 4 October 1992 from and 8-year old boy diagnosed in the Armed Forces Hospital, Khamis Mushayt, Saudi Arabia, as having endocarditis. This isolate, sent by Dr P.B. Nielsen, Consultant Microbiologist there, was a non-capsulating *B. anthracis*. It was not definitely established, however, that this isolate was the cause of the endocarditis. (P.C.B. Turnbull, Centre for Applied Microbiology & Research).

Bison and anthrax in northern Canada

D.C. DRAGON[1], R.P. RENNIE[1] and C.C. GATES[2]

[1]Department of Medical Microbiology and Immunology, University of Alberta Hospitals, 8440-112 Street, Edmonton, Alberta T6G 2J2, Canada. [2]Department of Renewable Resources, Government of the Northwest Territories, P.O. Box 390, Fort Smith, Northwest Territories X0E 0P0, Canada.

History

Anthrax was first diagnosed in northern Canada in 1952 when two employees of Wood Buffalo National Park (WBNP) were successfully treated for the disease after handling a bison *(Bison bison)* carcass. The first confirmed mass epizootic of the disease, however, did not occur until 1962 in the Hook Lake region of the Slave River Lowlands (SRL) (Figure 1).

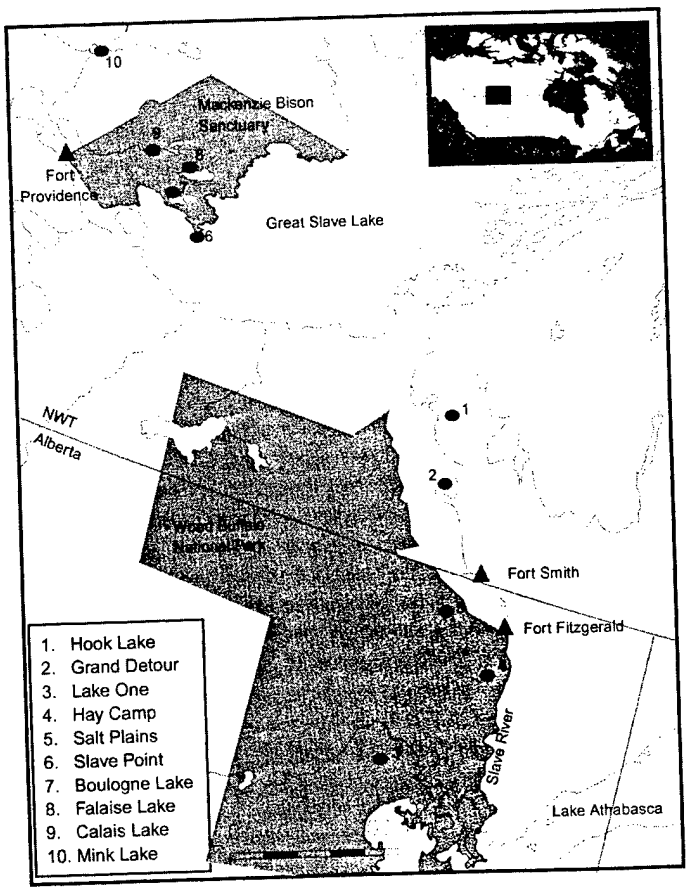

Figure 1. Areas of anthrax epizootics in bison in northern Canada from 1962 to 1993.

Since then the disease has been responsible for seven major epizootics among the northern bison herds resulting in the death of at least 1273 bison and several moose (*Alces alces*) (Table 1). During its sporadic summer epizootics, anthrax has extended its area of effect to include new regions of WBNP and SRL and several areas of the Mackenzie Bison Sanctuary (MBS) (Figure 1).

The origin of anthrax in northern Canada is unknown. After the first epizootic, it was hypothesized that horses which had died in the region may have carried *Bacillus anthracis* up from the south. However, the area where the horses had originally come from does not have a history of anthrax and, more importantly, the retrospective identification of the two WBNP human cases indicated that the organism was already present in the area a decade previous to the epizootic. Oral tradition and Hudson Bay Company records also indicate the presence of anthrax-like epizootics in the region's bison herds as far back as 1821. It has been hypothesized that migratory waterfowl may have transported spores from endemic areas in the southeastern United States. The affected areas of northern Canada all fall along the central migratory flyway of North America which originates in Louisiana and Texas, where it is believed that anthrax first came to land in the New World with the livestock of settlers.

Epidemiology

Anthrax epizootics in the bison herds have occurred between late June and early September during years when the area involved experienced a wet spring followed by a hot, dry summer. Because the meteorological conditions associated with "anthrax years" result in population explosions of biting insects, they have been implicated as vectors in the spread of anthrax in the bison. However, in every anthrax epizootic in northern Canada to date, mortality has been much higher among sexually mature bulls than females and immature bison. If insect vectors were involved in the perpetuation of anthrax epizootics, one would not expect such a discrimination.

The "anthrax season" corresponds roughly with the bison rut (Figure 2). The modified resistance hypothesis of Gainer and Saunders[2] states that drought, increased insect harassment and decreased feed during the summers associated with disease may act to alter the bison's immunity making them susceptible to low levels of anthrax spores that are ubiquitous in the environment. They state that breeding stress suffered by mature bulls during the rut helps to further reduce the bulls' immunity, accounting for their increased mortality. However, anthrax epizootics often begin before the rut proper at a time of year when the bulls are at their fittest (Figure 2)[3].

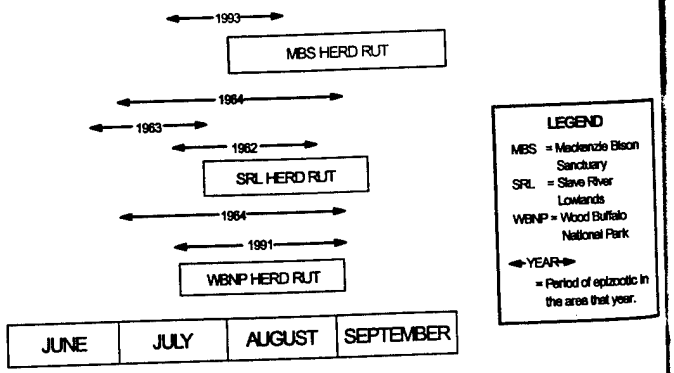

Figure 2. Occurrence of anthrax epizootics in northern Canada in relaion to the bison rut of the ares involved.

Scavenging of the carcasses is very common and aids in the dissemination of anthrax spores. Avian scavengers have been especially implicated in the dispersion of anthrax to new areas as spores have been recovered from the digestive tracts of herring gulls *(Larus argentatus)* and ravens *(Corvus corax)*. Except for three dead black bears *(Ursus americanus)* found during the 1993 MBS clean-up operations, no other scavengers have been found dead or suffering from the disease. *Bacillus anthracis* was not cultured from any of the bears and their actual cause of death remains unknown[3].

Table 1. Number of carcasses found by area during anthrax epizootics in the bison herds of northern Canada.

	Slave River Lowlands		Wood Buffalo National Park			Mackenzie Bison Sanctuary				
Year	Hook Lake	Grand Detour	Hay Camp	Lake One	Salt Plains	Falais Lake	Boulogne Lake	Slave Point	Calais Lake	Mink Lake
1962	281	-	-	-	-	-	-	-	-	-
1963	12	269	-	-	-	-	-	-	-	-
1964	44	202	46	7	-	-	-	-	-	-
1967	-	-	-	120	-	-	-	-	-	-
1968	-	-	-	1	-	-	-	-	-	-
1971	37	-	-	-	-	-	-	-	-	-
1978	12	27	40	-	-	-	-	-	-	-
1991	-	-	-	-	32	-	-	-	-	-
1993	-	-	-	-	-	111	26	9	23	3

Ecology of spores

Early studies on the ecology of anthrax found a correlation between the occurrence of disease and specific soil factors, such as alkaline pH, high moisture content, and high organic content. Based on these correlations, Van Ness[7] postulated his "incubator area" hypothesis which states that in low lying areas, under conditions of alkaline pH, high soil moisture and the presence of organic matter, *B. anthracis* may undergo cycles of spore germination, multiplication and resporulation. Van Ness believed the soil factors would have little influence on static spores and therefore must be affecting vegetative cells present in the soil. Experimental evidence, however, indicates that the vegetative cells of *B. anthracis* have very specific nutrient and physiological requirements and survive poorly outside a host or artificial medium[5]. Experimental germination of *B. anthracis* in the environment has only been successful in soil or water that has been artificially enriched with animal blood or viscera. Even when adequate nutrients are provided, the vegetative cells have proven very susceptible to antagonism from other bacteria species present. Such antagonism leads to an overall decline in the number of anthrax spores present even when other conditions favour growth[6].

A review of the properties of spores of *B. anthracis* and other *Bacillus* species indicates that spores while dormant are not static and are influenced by their environment[1]. Specific soil factors correlated with the disease may aid in the concentration and maintenance of viable anthrax spores. High levels of calcium in the alkaline soil may help to maintain spore vitality for prolonged periods of time. In the laboratory, calcium has proven important in the maintenance of dormancy and resistance of *Bacillus* spores. *Bacillus* spores have a high buoyant density and anthrax spores could be collected from the surrounding area into low lying areas by cycles of runoff and evaporation. Low-lying depressions may act as "concentrator areas." These areas would be the last to dry out (high moisture content) and would also collect other floating organic debris from the surrounding area.

Bison behavior

It is possible that the predisposition of bison bulls to anthrax may be due to selective exposure to spores through gender-specific behavior. Even before the rut proper begins, bulls begin to display aggressive behavior towards one another in order to establish dominance. Such aggressive behavior includes increased wallowing, pawing and charging which creates large clouds of aerosolized dust around the bulls. Bison wallows are employed year after year by bulls and, because of the repeated use, often represent low lying depressions on otherwise level meadows. Anthrax spores concentrated into wallows may be aerosolized by bulls, enter their respiratory tract through either normal respiration or snorting, another common rutting behavior, and thereby establish disease.

Alternatively, Komers[4] has recently observed that bulls and cows display different feeding patterns. Cows are more selective than bulls, feeding on higher, new-growth vegetation. Bulls, however, graze the plants to the ground. The bulls, therefore, spend more time in closer contact with potentially contaminated soil than the cows.

Conclusions

We suggest that in northern Canada, cycles of runoff and evaporation act to move anthrax spores previously dispersed in the area by scavengers, into low lying concentrator areas. Calcium present in the alkaline soil of the concentrator areas may act to greatly extend the viability and, therefore period of infectivity, of the spores. During drought conditions gender-specific feeding or breeding behavior of bison bulls selectively exposes them to lethal doses of anthrax spores in the concentrator areas, resulting in an epizootic.

Acknowledgements

The authors thank Dr B.T. Elkin and the Departments of Renewable Resources and Student Services, Government of the Northwest Territories, for their continued support. The authors acknowledge Judy Dragon for her help with Figure 1. Dan Dragon is the recipient of a Mary Louise Imrie Graduate Student Award from the Faculty of Graduate Studies, University of Alberta, which has aided in making attendance at this workshop possible.

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A review of anthrax in the Etosha National Park

P. M. LINDEQUE[1], C. BRAIN[1] and P. C. B. TURNBULL[2]

[1]Etosha Ecological Institute, P.O. Okaukuejo, via Outjo, Namibia. [2]Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, UK

(This paper is a summary of work published elsewhere)

Anthrax was first diagnosed in Etosha National Park in 1964, although there is evidence to suggest that the disease was present in the region long before that[1]. The annual incidence of anthrax became relatively high in the years 1968-1972, and since then the incidence has remained consistent but low, except for two intense outbreaks in elephants *(Loxodonta africana)* in 1981 and 1989, and one localized outbreak affecting mainly Burchell's zebra *(Equus burchelli)* and blue wildebeest *(Connochaetes taurinus)* in 1984 (Fig. 1A). The increase in elephant anthrax mortalities seems to have coincided with the dry rainfall cycle experienced over the past 15 years in the region (Fig. 1B).

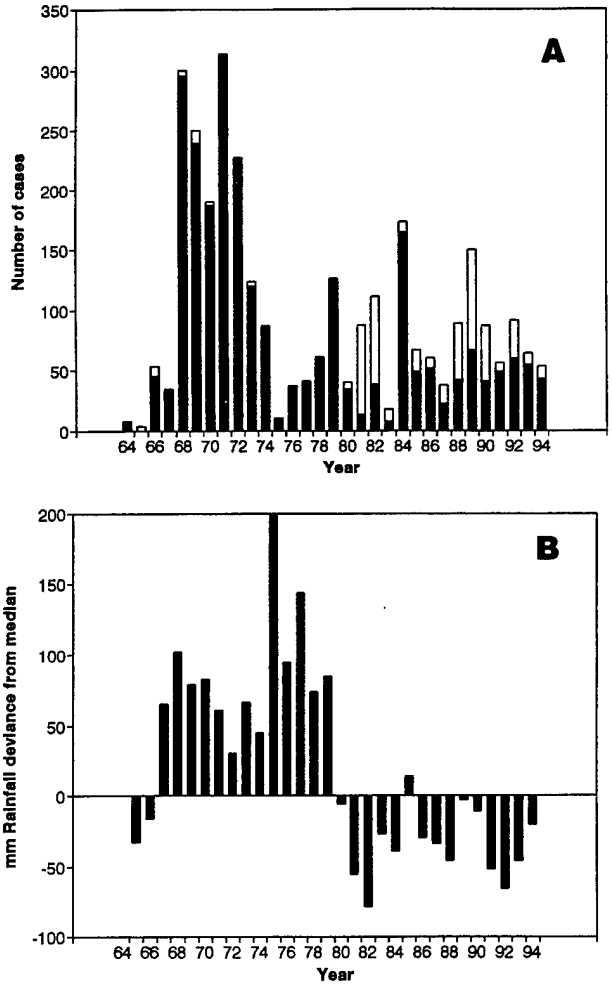

Fig 1. A Anthrax mortalities in Etosha National Park ☐ elephants; ■ all other species);
B. 3-year running mean minus long term median (330.2 mm) rainfall for Okaukuejo, Etosha National Park.

The overall spatial and temporal distribution of anthrax mortalities in the plains ungulate species has not changed greatly since anthrax was first diagnosed. Dr Ebedes had described enzoötic areas in central and eastern Etosha, which to this day remain the foci of repeated annual outbreaks (Fig 2.). The seasonal pattern of anthrax mortalities in the plains ungulates still shows a peak in the months of March and April, coinciding with the end of the wet season (Fig 3)[2].

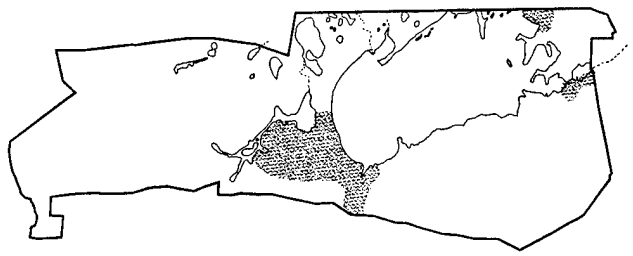

Fig 2. Anthrax enzoötic areas (hatched) in Etosha National Park, as described by Ebedes (1976).

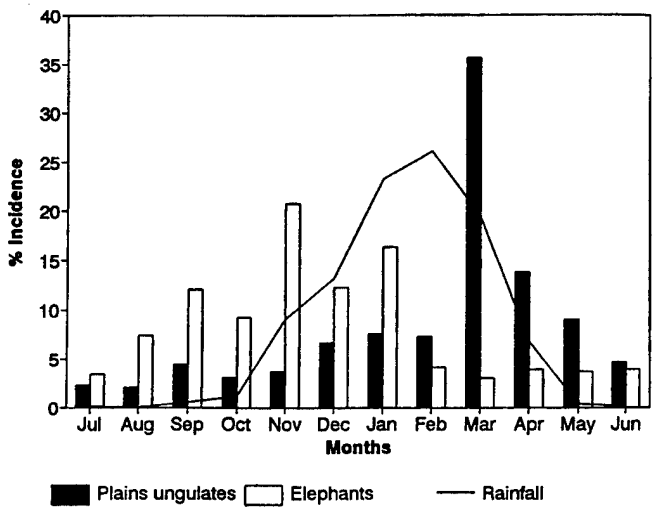

Fig 3. Monthly incidence of anthrax mortalities and rainfall in Etosha National Park.

The outbreak in elephants in 1981 seems to have marked a change in the incidence in this species. Since the outbreak in 1981, the annual incidence of anthrax in elephants has remained higher than previously recorded. Furthermore, anthrax mortalities in elephants show a different spatial and temporal pattern to the other affected species. Elephant anthrax mortalities occur mostly towards the end of the dry season. Spatially, elephant anthrax mortalities are not restricted to the enzoötic areas previously described, but occur throughout the park, with a high incidence in the western area. The spread of the disease to the west seems to have coincided with the outbreak in elephants during 1981, and is probably due to the ability of elephants to move large distances over short periods, thus spreading the disease. It is interesting that despite anthrax being present in the west of the park for the past 14 years, very few cases in species other than elephant have occurred (Fig 4.).

The exact manner in which wildlife species contract anthrax in the wild is unknown, but it is assumed to be through ingestion of contaminated material. Another unknown is the necessary level of exposure, ie. is it a few spores or millions. It is very probable that this is dependent on a number of other factors. We tried to identify places in the environment where high concentrations of anthrax spores could be found. Because earlier studies identified artificial waterholes and gravel pits as potential sources, we tested water and soil from different water types ten times over a three year period (Table 1). The results showed the gravel pits did seem to

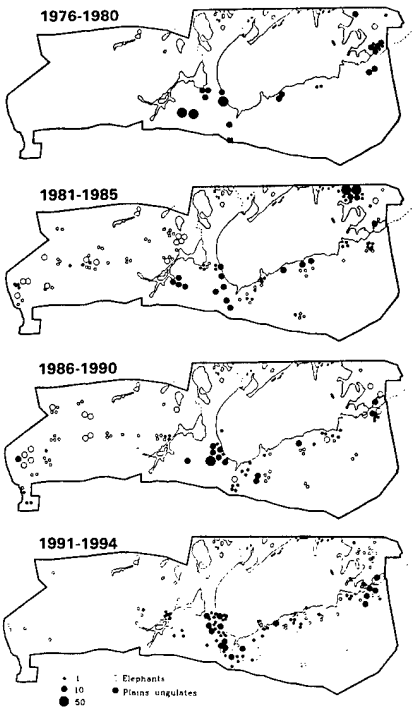

Fig 4. Distribution of recorded anthrax mortalities in the Etosha National Park.

have a higher incidence overall, but most of the isolations came from the same gravel pit, and one of the concentrations can be considered very high. Laboratory experiments done using water from different water sources did not support the theory that gravel pits, or any other water source in Etosha, are 'incubator areas'. Vegetative *B. anthracis* inoculated into water samples either died out or sporulated, but there was no evidence that they multiplied unless a substantial amount of extra nutrients were added to the water [2]

Table 1. Isolation of anthrax spores at water sources in Etosha National Park

Category	Number Tested	Number Positive	Concentration Range (CFU/g or ml)
Natural Fountains	**5**	**1 (20%)**	
Repetitions:			
Soil	50	1 (2%)	10/g
Water	32	0 (0%)	
Boreholes	**5**	**2 (40%)**	
Repetitions:			
Soil	50	1 (2%)	80/g
Water	32	1 (3%)	1/ml
Gravel Pits	**9**	**2 (22%)**	
Repetitions:			
Soil	90	4 (4%)	8-80/g
Water	23	2 (9%)	1/ml
Natural Pans	**4**	**1 (25%)**	
Repetitions:			
Soil	40	1 (3%)	4/g
Water	5	0 (0%)	

Terminal *B. anthracis* counts were determined whenever an animal was found soon after death (Table 2). Looking at these, it becomes evident that the potential for contamination at the site of a carcass is great. Contamination levels at carcass sites, however, were seldom $>10^5$ spores per gram, and more often were $<10^3$. Experiments were performed contaminating different soils with blood from an anthrax case, and following the total and spore *B. anthracis* count over time. These experiments showed that soil can greatly influence the degree and success of sporulation (Fig 5.). It was also found that high concentrations of anthrax spores around a carcass are limited to a radius of about five metres, beyond which recovery rate is low and scattered. Carcass sites, however, seem to be the major source of high concentrations of spores in the environment, and anthrax spores at these sites were found to be capable of surviving for at least 82 months (the longest period examined so far) in the Etosha environment. Another identified source of high anthrax spores concentrations was found to be scavenger faeces, with hyaena showing the highest levels, and vultures the lowest. The result of this survey seem to reflect the habits of the species involved, as vultures, that normally feed on a relatively fresh carcass, are less likely to ingest spores whilst hyaenas may chew on bones and skin highly contaminated with spores.

Table 2. Viable *Bacillus anthracis* counts (cfu per ml) at time of death in the blood of animals that died of anthrax in the Etosha Park.

Species	Number tested	Mean terminal viable count per ml	Range
Burchell's zebra	11	2×10^8	3×10^6 - 7×10^8
Elephant	6	3.7×10^8	330 - 2×10^8
Springbok	2	1.2×10^8	2.5×10^8 & *5×10^5
Blue wildebeest	2	7×10^7	1×10^8 & †4×10^7
Cheetah	1	5×10^8	

*Pleural fluid count = 3.5×10^6 cfu/ml † Peritoneal fluid count = 2×10^5 cfu/ml

A serological survey, using an ELISA test, showed that 98% of lion, hyaena and jackals in Etosha have very high antibody levels against one of the anthrax toxins, protective antigen (PA). In contrast, only 7% of all herbivores tested showed any degree of anti-PA antibodies, suggesting that few survive an anthrax infection.

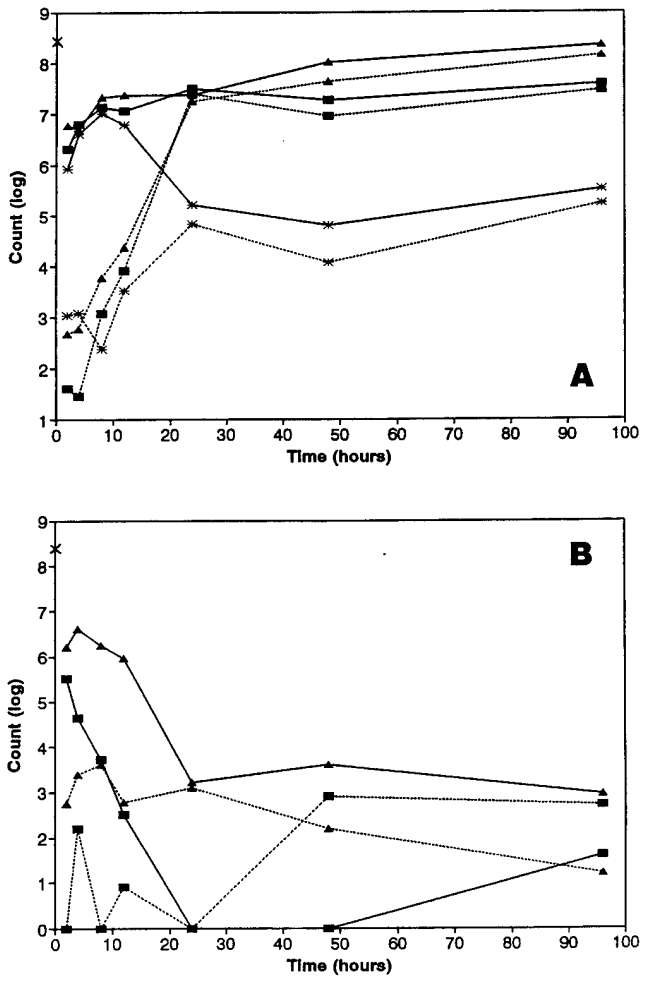

Fig 5. Fate of *Bacillus anthracis* within blood and mixed with sandy soils (A) and Karstveld type soils (B). (Solid line = total aerobic count, broken line = spore count, X = contamination level).

In the early 1960s, cheetah were more common in the Park than in recent times[1]. In 1970[1] reported two suspected anthrax cases in cheetah, and speculated that anthrax may have played a role in the decline in numbers of this species. In a recent study on cheetah, a total of seven individuals were radio-collared in an 18 month period. Six of these died, and five were confirmed to have had anthrax (the last was a suspected case). This seems to support the idea that perhaps in Etosha the cheetah population is limited by anthrax. Of eight cheetah sera tested for antibodies to anthrax, weak positives were only obtained in three. This again reflects a habit of the species; cheetah rarely scavenge and therefore are less exposed to anthrax carcasses than other carnivores.

Table 3. *Bacillus anthracis* spore count levels in 165 soil samples associated with anthrax carcasses in the Etosha National Park.

Count/ml	Number of result
1 - 10	35
11 - 10^2	49
101 - 10^3	38
1001 - 10^4	22
10001 - 10^5	14
100001 - 10^6	6
> 10^6	1

Table 4. Results of a survey on the presence of anthrax spores in the faeces of black-blacked jackals, vultures and hyaenas in the Etosha National Park.

Species	Number Tested	Number Positive	Mean Count/g	Range
HYAENA				
In vicinity of carcase	5	3 (60%)	6740	2 - 20000
Away from a carcase	1	0 (0%)		
JACKAL				
In vicinity of carcase	25	18 (72%)	626	2 - 4480
Away from a carcase	9	0 (0%)		
VULTURE				
In vicinity of carcase	18	9 (50%)	34	2 - 174
Away from a carcase	4	0 (0%)		

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Anthrax in the Kruger National Park: temporal and spatial patterns of disease occurrence

V. DE VOS and HELENA B. BRYDEN

National Parks Board, Skukuza, Kruger National Park, South Africa.

Summary

It appears that in the Kruger National Park, a two million ha natural system, anthrax reacts and conforms to intrinsic governing factors, creating identifiable and predictive temporal and spatial disease distribution patterns.

Temporal distribution patterns: Indications are that anthrax epidemics in the KNP have a periodic or cyclic pattern of occurrence. So far, the intervals between major epidemics appear to be in the order of 10 years or multiples thereof. Long term climatic changes also occur roughly in cycles of 10 years, and although no direct correlation could be found, it is speculated that climatic changes could have an effect through kudu *(Tragelaphus strepsiceros)*, the principal host of anthrax in the KNP. A distinct clustering of disease events per season was also found. Anthrax occurs almost exclusively during the dry late winter and early summer months and is invariably interrupted or terminated by the onset of rain. It was found that in all anthrax epidemics the distribution of the incidence of disease events (mortality) form typical propagated epidemic curve patterns extending over a few months. This not only points to a short cycle type of infection, but also indicates that anthrax is density dependent in the KNP.

Spatial patterns: The distribution of the disease events in time (yearly) and space (KNP) indicate that anthrax occurs endemically in Pafuri, the northern-most tip of the KNP, and in periodic epidemic form in the rest of the KNP, with a distinct predilection for the northern areas. An analysis of the spreading characteristics of individual anthrax epidemics in the KNP revealed that anthrax epidemics move slowly initially, then pick up momentum to move almost relentlessly, like a runaway bush fire, until factors such as natural barriers (e.g. a flowing river), a lack of susceptible hosts in a specific area or rain stops it progress. The fore front of the infection has the tendency to move unidirectionally, with occasional "mopping up" of some individuals which escaped the main onslaught, in the area behind. The 1990/91 epidemic is described as a typical example.

Introduction

Although there has been a dramatic decline in anthrax outbreaks in commercial livestock farming areas, anthrax remains an important disease in wildlife in free-ranging situations. Major epidemics have been, and periodically still are, recorded in wildlife conservation areas such as the Ruwenzori National Park in Uganda, the Selous Nature Reserve in Tanzania, the Etosha National Park in Namibia, the Luangwa Valley in Zambia, and the Kalahari Gemsbok, Vaalbos and Kruger national parks in South Africa. (Choquette and Broughton, 1981; De Vos, 1990; De Vos and Lambrechts, 1971; De Vos *et al*, 1973; Ebedes, 1977; Ebedes, 1981; Gainer, 1987; Pienaar, 1960, 1961, 1967; Turnbull, 1986; Turnbull *et al*, 1991).

In the Kruger National Park (KNP) anthrax was first observed and diagnosed in 1959 (Pienaar, 1960). Since then anthrax has occurred regularly in the KNP, with certain indications of a clustering of disease events in time and space emerging. The KNP is managed according to a policy of minimal interference in the natural processes of the ecosystems, of which indigenous diseases are considered integral and necessary elements. This provides excellent opportunities to establish normals and baseline knowledge for anthrax in an essentially natural setting.

The aim of this report is to identify and quantify patterns for the temporal and spatial occurrence of anthrax in the KNP.

Materials and methods

The study area was the KNP, an elongated conservation area consisting of two million ha subtropical savanna woodland, between 20°19' to 25°32' latitude south and 31°0' to 32°02' longitude east, situated in the north-eastern most corner of

South-Africa. It borders on Mozambique in the east and Zimbabwe in the north. Commercial farms, traditional communal grazing areas and private nature reserves are present along the western and southern boundaries. The entire area is fenced and contains ± 2,332 kg biomass of large mammals per square km, made up of 20 different species.

The outbreaks were closely monitored for six days per week by ground vehicle teams and since 1970 by a helicopter team as well, all actively looking for carcasses. These teams located the carcasses, mostly by monitoring for vulture activity and collected the necessary samples for diagnostic purposes.

Blood smears that were routinely taken, were stained with Giemsa stain and examined for *Bacillus anthracis* organisms as described by Parry *et al* (1983). Some faecal samples from vultures, soil, water, sediment, bone and other tissue samples were collected and subjected to bacteriological examinations. Uncontaminated fresh specimens were cultured on serum and blood agar, whilst contaminated material such as soil, faeces, bone, etc. were cultured on PLET selective medium of Knisely (1966). Diagnosis was made, taking into account factors such as history, clinical evidence where available, appearance of carcass, smear examinations, culture and bacteriological characteristics and pathogenicity in laboratory animals. Laboratory identifications were made in accordance with tests as proposed by Parry *et al* (1983).

Results and discussion

Temporal patterns

There appears to be cyclic pattern in the occurrence of major anthrax epidemics in the KNP (Fig. 1). The reason for this periodicity is not clear, although circumstantial evidence points to the fact that rainfall cycles are in some way involved. As depicted by Figure 1, both seem to occur roughly in cycles of 10 (rainfall) or multiples of 10 years (anthrax epidemics). Although very little correlation between the two could be found, it is assumed that the rainfall pattern somehow affects the long term anthrax cycle, maybe indirectly through the host population or environment. As dipicted in Figure 4, anthrax epidemics are dependent on a certain density of the kudu *(Tragelaphus strepsiceros)*, which in turn is dependent on the climate (De Vos, 1990).

Epidemic outbreaks also conform to a distinct pattern which is related to season and more specifically rainfall. As depicted by Figures 2 & 3, all anthrax outbreaks occurred during the dry season in the latter part of the winter and were all interrupted by the start of the rainy season during the early summer of each year.

As depicted by Figure 3, the northwards spread of the 1990/91 outbreak was interrupted by rain during the summer months of November 1990 to April 1991. The same was again seen in November of 1991 when the epidemic finally ended. Untimely rain for the season fell in May/June and again in September of 1991, followed by a temporary decrease in mortalities, as reflected by the epidemic curve (Fig. 4).

Rainfall therefore has a vital effect on the frequency of occurrence and spread of anthrax in the KNP. Depending on the intensity and volume of rainfall, it will either suppress or stop an outbreak completely, due to several reasons. Rain effectively washes infection from vegetation, acts as a diluting factor and as a vehicle to take infective material to the low lying areas and into fast flowing rivers and eventually out of the system. It fills up water holes and provides natural open water, resulting in dispersal of the high densities of animals which build up around permanent water holes during the dry months. It also provides vultures with alternative bathing areas.

In all the major anthrax epidemics in the KNP the occurrence of new disease events (mortalities) in relation to time were found to rise and then fall, taking on the approximate form of the familiar statistical bell-shaped distribution curve (Fig. 4). Without exception these are typical propagated epidemic curves as described by Schwabe *et al* (1977), pointing to a short cycle type of infection where disease is transmitted directly or indirectly from infected hosts, giving rise to an initial relatively slow multiplication phase, followed by an exponential growth phase, illustrated by a dramatic rise in the incidence of new cases. It also demonstrates that as the epidemic continues, those infected individuals either die or recover, with the result that the supply of susceptibles becomes depleted. As a consequence the epidemic tapers off relatively slowly and finally stops. Although the epidemic curves of all major anthrax epidemics in the KNP are seen to terminate prematurely as a result of rain, indications are that tapering off has already started before the commencement of rain (Figs. 2, 3), illustrating that the disease is density dependent and self-limiting in a natural environment, such as the KNP.

Spatial patterns

As depicted by Figure 5, the spatial distribution of anthrax in the KNP, over a 35 year period (1959 to 1994), shows a distinct predilection for the northern areas of the KNP; the further north, the higher the frequency of occurrence seem to be. Since 1959 anthrax has occurred in 19 out of a possible 35 years in Pafuri, the northern-most tip of the KNP, which is considered to be within the realms of predictability, giving this area endemic status.

Anthrax is mostly contained within this endemic area by its geographical position, which is low-lying (c 200 m above sea level), rising up southwards to 400 m over a distance of *ca* 10 km. This transitional area is rugged, well drained, with a low animal density and serves as an effective barrier to the southward spread of anthrax. As explained earlier, the rest of the KNP is ravaged by periodic major anthrax epidemics.

All epidemics in the KNP spread slowly initially, then pick up momentum to move almost relentlessly, like a runaway bush fire, until barriers (eg. a flowing river or fence), a lack of susceptible host or rain stops its progress. The 1990/91 epidemic illustrates this principle very well (Fig. 6). From the initial focus of infection the 1990/91 outbreak initially spread in an easterly and southerly direction. The perennially flowing Olifants Rivers checked the southward spread, and the outbreak then turned northwards. The stagnant pools in the Letaba River and its tributary actually accelerated its northwards spread and artificial water holes formed a further link of foci of infection right up to the Shingwedzi River (Fig. 6).

In a flowing river such as the Olifants, infective material (anthrax spores), carried there chiefly by vultures, are continuously "diluted" and washed away. A running stream also provides water over a wide front, with less concentration of animals at specific points. Although passable, it provides a barrier to the free movement of animals across the river. Stagnant pools and artificial water holes, on the other hand, allowed for concentrations of host animals (local overabundance) during the dry period, with better chances of disease transmission taking place. Being the assembly point of not only host animals, but also scavengers such as vultures, hyenas and lions, the chances of infection being concentrated at these points are excellent, and being stagnant, the infection is not washed away and builds up in these pools.

In an effort to provide water during the dry period of the year, artificial water holes with windmills were erected in areas where animals previously would not have concentrated during the dry season. There was also a tremendous increase in the number of boreholes with artificial drinking troughs during the

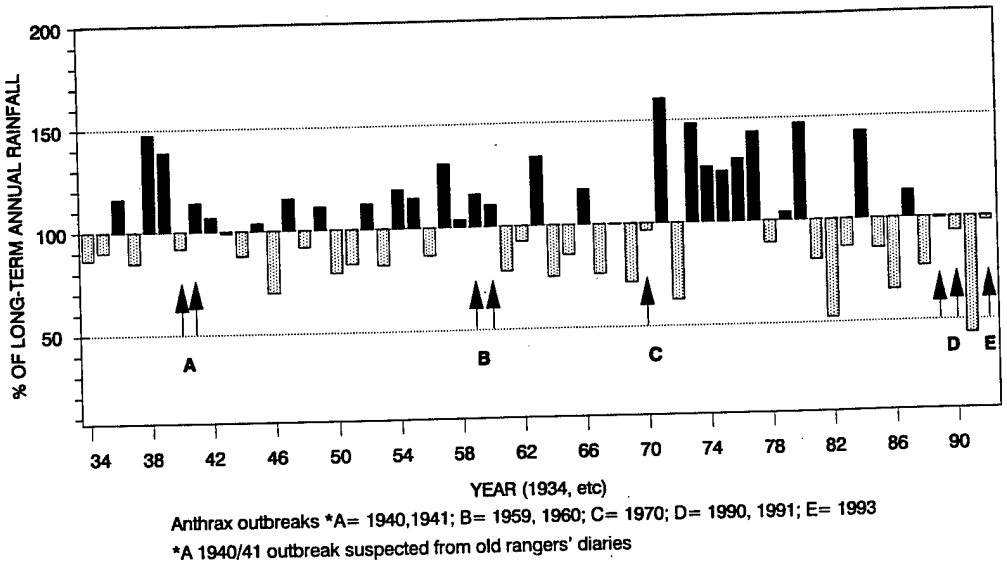

Figure 1. Cyclic occurrence of anthrax epidemics in relation to percentage deviation from long-term mean annual rainfall for the northern part of the KNP.

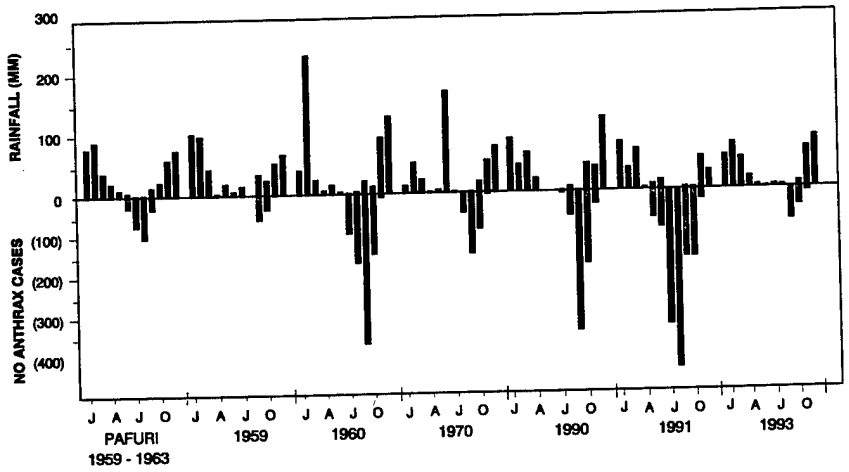

Figure 2. The occurrence and relations between the major anthrax epidemics and rainfall in the KNP.

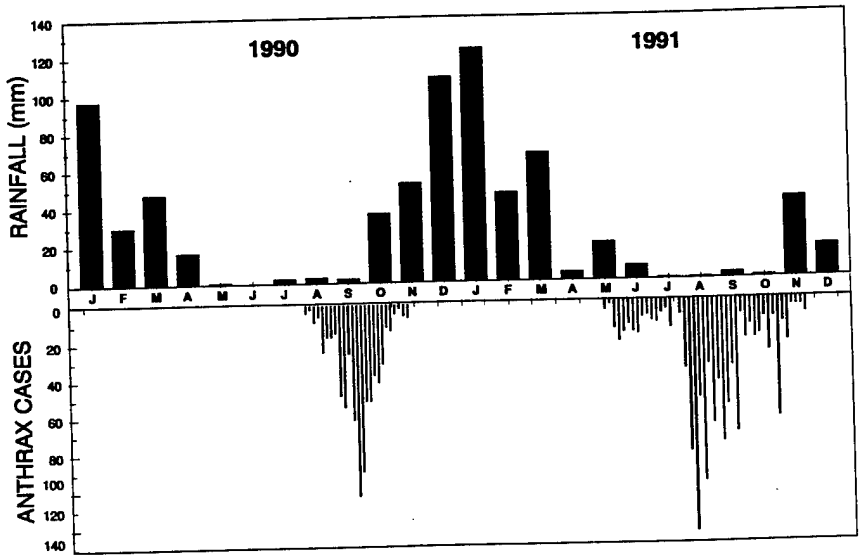

Figure 3. The occurrence of anthrax per four day intervals in relation to monthly rainfall in the northern region of the KNP during the 1990/91 epidemic.

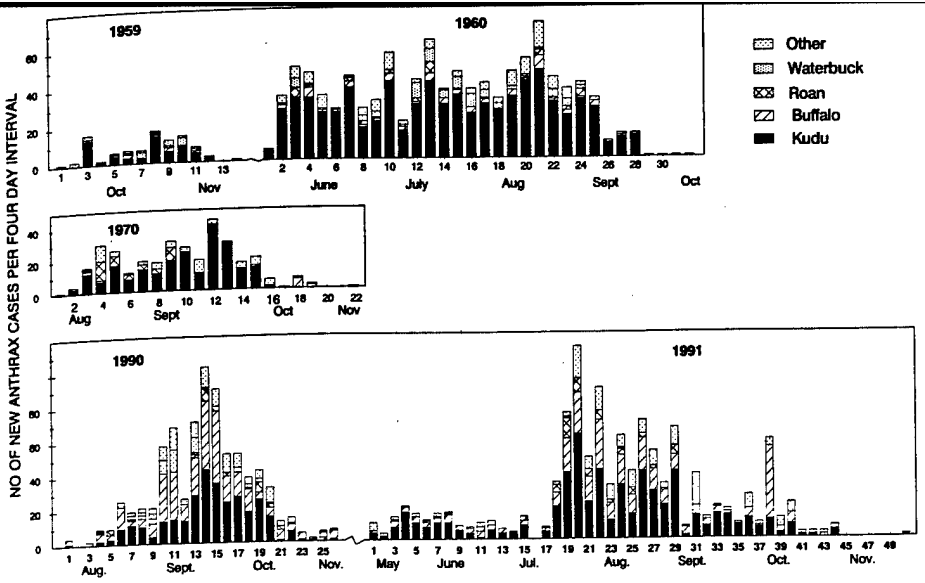

Figure 4. Epidemic curves of the different anthrax outbreaks in the KNP.

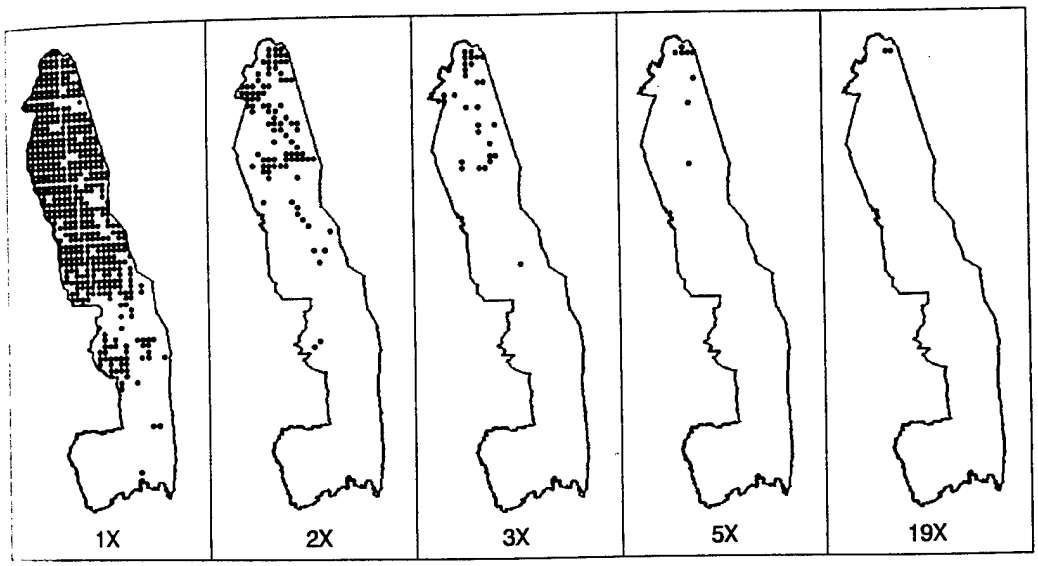

Figure 5. The frequency of occurrence of anthrax for the period 1959-94. One or more occurrence per locality within the same year is treated as a single occurrence.

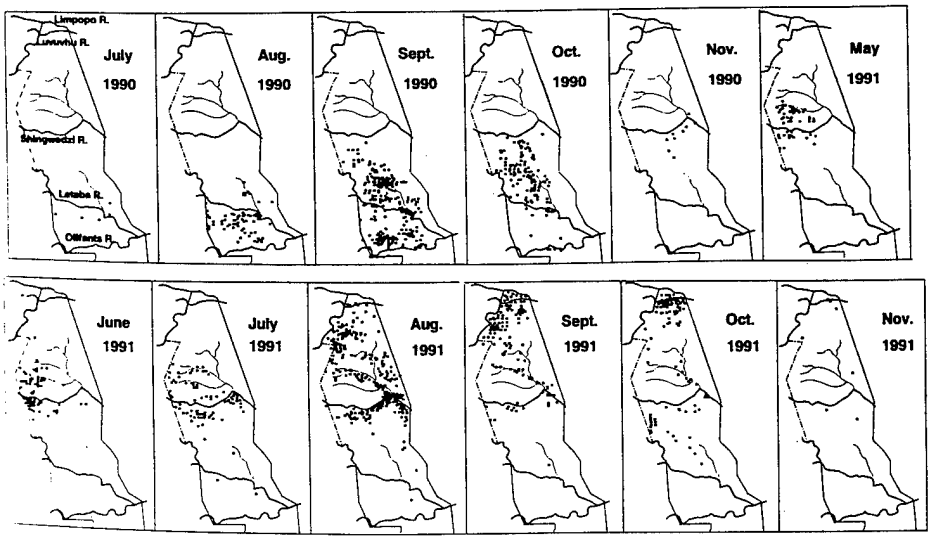

Figure 6. The pattern and spread of anthrax during the 1991/92 epidemic in the KNP. The spatial distribution of the number of new cases per calendar month is shown.

1970s; from 140 in 1969/70, to 320 in 1979/80. These provided a link between the Letaba and Shingwedzi rivers and further northwards to the Luvuvhu/Limpopo rivers. These water holes also proved to be much sought-after drinking and bathing areas for vultures.

The outbreak formed a fore-front, which can also be described as the bulk, or main part of the outbreak. This bulk, slowly but surely spread northwards, with some early cases occurring ahead and some sporadic cases ("mopping" up of individuals that escaped the initial onslaught) still occurring behind. At the height of the outbreak in October 1990, the main part of the outbreak was located centrally, with virtually the whole area between the Olifants and Shingwedzi rivers infected (Fig. 6). In November, with the onset of the rainy season, the outbreak faded out temporarily at the level of the Shingwedzi River. Up to that point the outbreak had been virtually limited to the granitic soils of the western side of the KNP, with only a few cases on the eastern basaltic flats. This was due to a general shift of animals from the eastern Mopani Shrubveld to the more nutritious and water rich western granitic, mixed Mopani/*Combretum/Acacia* landscapes. Most of the water was also present on the western side.

The outbreak flared up again in May of the following year (1991), stayed briefly in the region of the Shingwedzi River, and then moved northwards and eastwards. It again followed a dry river bed with stagnant waterpools (the Mphongolo tributary) and artificial water holes northwards across the basaltic plains, over the Luvuvhu River and finally faded out in November against the Limpopo River. Strong signs of the fading out phase (downward curving of the epidemic curve) was already apparent before the rain started (Fig. 4).

Significantly the body of the outbreak consistently progressed unidirectionally and never showed any signs of doubling back southwards in spite of the fact that there were still thousands of susceptible individuals in the area. This serves as further corroboration of the fact that the disease is density dependent and self-limiting.

The same basic pattern was seen during the 1959/60 anthrax epidemic, with the difference that the outbreak progressed and spread from north to south, with hydrogeographical features combined with low density animal areas providing temporary barriers along the way. This created a three pronged epidemic curve (Fig. 4).

Those patterns that were established is considered accurate enough to be used to predict the behaviour, course and outcome of future anthrax outbreaks and control measures in the KNP.

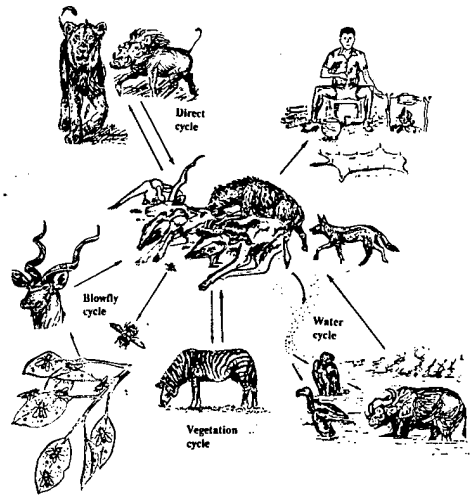

Figure 7a. Epidemiological anthrax cycle in the KNP. Anthrax follows two major epidemiological routes in the KNP: through water via vultures and other scavangers, or through infected vegetation via blowflies.

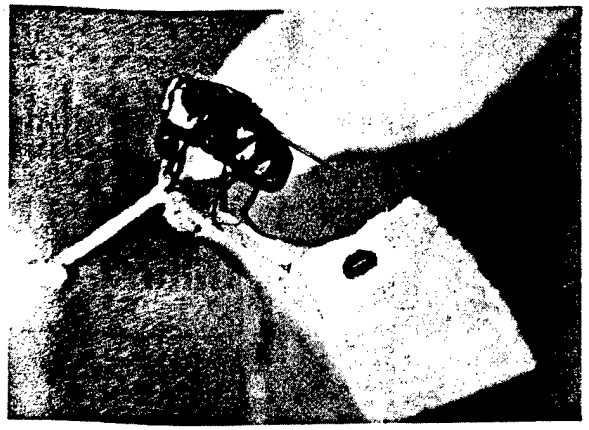

Figure. 7a. A blowfly with vomit droplet on leaf. Blowflies, m *Chrysomya albiceps* and *C. marginalis*, feed in large numbers on the fluids from infected carcasses, and then deposit vomit droplets on vegetati the immediate vicinity. *B. antracis* thus deposited is ingested by browsers as kudu.

Acknowledgements

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A survey of worldwide strains of *Bacillus anthracis*

PATRICIA F. FELLOWS

Bacteriology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21702-5011, USA

The views, opinions and/or findings contained in this publication are those of the author and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

Summary

Thirty-one isolates of *Bacillus anthracis* were selected from the Bacteriology Division culture collection of the United States Army Medical Research Institute of Infectious Diseases and examined for phenotypic and biochemical characteristics, as well as for plasmid content. In addition to standard laboratory strains, isolates collected from human and animal sources from various worldwide locales were also included. The intent of this study was to establish a catalog of well-described, diverse isolates.

Material and methods

Strains

The *Bacillus anthracis* isolates used in this study are listed in Table 1.

Media

Brain heart infusion broth (BHI, Difoo Laboratories, Detroit, MI) was used for culturing *B. anthracis* isolates. Tryptic soy agar (TSA, Difco Laboratories) or 5% sheep blood agar (SBA) was used to define colony morphology. Protease detection medium was prepared according to Aronson[1] and contained casein as the protease substrate. Hemolysin agar was prepared by using defibrinated sheep blood that had been washed five times with sterile phosphate-buffered saline (PBS) containing calcium and magnesium (BioWhittaker,Walkersville, MD). The washed sheep blood cells were resuspended in PBS, which contained 1/100 volume of a 0.2 M stock of dithiothreitol (DTT), before they were added to blood agar base (Difco Laboratories). Immunoassay agar plates contained 12 ml of R agar[9] plus 1 ml of goat anti-protective antigen (PA) serum. Bicarbonate agar plates were prepared from BHI supplemented with 10% horse serum (GIBCO Laboratories, Grand Island, NY) and 0.8% sodium bicarbonate.

Phenotypic characteristics

B. anthracis isolates were inoculated onto SBA, hemolysin agar, TSA, and protease detection medium and incubated overnight at 37°C to develop phenotypic characteristics. The presence of PA and capsule production was assayed by plating cells on immunoassay agar and bicarbonate agar, respectively. The plates were incubated overnight at 37°C with 5% or 20% CO_2.

Sensitivity discs

Antibiotic sensitivity was determined by plating cells for confluent growth on SBA, adding a penicillin sensitivity disc (10 units; Becton Dickinson and Company, Cockeysville, MD) to the surface of the agar and incubating overnight at 37°C[8].

Sporulation

Sporulation was evaluated by plating cells onto SBA. After incubating overnight at 37°C, the cultures were incubated at room temperature (about 25°C) for 3 days. A loopful of growth was suspended in PBS, placed on a glass slide, and viewed under phase contrast microscopy for the presence of spores.

Genetic analysis

A modification of procedures described by Kado and Liu[5] was used for plasmid isolation. The polymerase chain reaction (PCR) was used to probe plasmid preparations of the isolates for the presence of toxin and capsule genes. Oligonucleotide primers (Table 2) specific for these virulence determinants were provided by F. Knauert and T. Hoover (USAMRIID). Reagents used in the amplification process were those commercially available (Gene-Amp, Perkin Elmer Cetus, Norwalk, CT). Amplification of the DNA was carried out by using procedures described in the product literature accompanying a Perkin Elmer Cetus DNA Thermal Cycler. Samples were electrophoresed by using either a BRL mini-gel apparatus or BRL Model MPH apparatus (Bethesda Research Laboratories, Bethesda, MD).

Results

For comparison purposes, results are listed in Table 1.

Colony morphology

Typically, *B. anthracis* colonies are rough, exhibit a slightly raised surface with a cut-glass appearance, and are greyish-white. Virulent strains often exhibit outgrowths from the colony, referred to as the medusa head appearance[8]. The poly-D glutamic acid capsule is evident as a smooth colony morphology in strains possessing the pX02 plasmid when grown on bicarbonate agar and incubated in the presence of CO_2. Typical strains do not produce capsule in air.

Although most strains exhibited the typical rough colony morphology, some variability was observed in this study. Isolates BA0001, BA0002 and BA0018 exhibited colonies approximately one-fourth the size of a typical *B. anthracis* colony. Two isolates, BA0006 and BA0052, exhibited colonies which appeared slightly shiny on TSA in the absence of bicarbonate and CO_2. Of special interest was isolate BA0046, which exhibited two colony types. One resembled *B. anthracis*, while the other was smooth, suggesting the presence of capsule in air on both SBA and TSA. The presence of capsule was verified by microscopic examination of the bacilli using India Ink. However, staining with fluorescent monoclonal antibodies specific for anthrax poly-D-glutamic acid capsule did not indicate *B. anthracis*.

Protease detection

B. anthracis produces an extracellular protease which appears to be most active during the log phase growth. This is evident by the lack of PA activity in cultures incubated beyond 30 h[11]. The presence of protease was identified by the hydrolysis of casein in the medium clearing a zone around the colony. Only three isolates, BA0001, BA0002 and BA1003 failed to produce protease by this method.

Table 1

Identification Number	Strain Name and Origin	Colony Morphology	Plasmids pX01	Plasmids pX02	Capsule Production Air	Capsule Production 5% CO_2	Capsule Production 20% CO_2	Immunoassay	Hemolysin	Protease	Sporulation	Penicillin Sensitivity	Toxin Genes PA	Toxin Genes LF	Toxin Genes EF	Capsule Genes A	Capsule Genes B	Capsule Genes C
	Animal Origin																	
BA0002	Pakistan (caprine) [a]	A	+	-	-	-	-	-	+	-	-	+	+	+	+	-	-	-
BA0006	Pakistan (caprine) [a]	A	+	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-
BA0018	Canada (bison) [a]	A	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA0019	Canada (bison) [a]	N	-	-	-	-	-	+	+	+	-	+	-	-	-	-	-	-
BA1002	Vollum 1B (variant of Vollum) [b]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1003	Vollum (bovine) [c]	N	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+
BA1004	Ames, USA (bovine) [d]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1022	Haita (caprine) [a]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1024	Ireland (wool) [a]	N	+	+	-	+	+	+	+	+	+	+	-	-	-	+	+	+
BA1031	South Africa (wildebeest) [a]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1034	Nebraska, USA (bovine) [c]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-
BA1043	South Africa, Sterne [e]	N	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
BA1050	Texas, USA (bovine) [d]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
NG	Texas, USA (ovine) [d]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	Human Origin																	
BA0001	Arkansas, USA [f]	A	+	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+
BA1008	New Hampshire, USA [g]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1023	California, USA [a]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1040	Colorado, USA [a]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-
BA1081	Zimbabwe-3 [i]	N	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
BA1083	Zimbabwe-2 [i]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1086	Zimbabwe-1	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1087	Scotand [i]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1099	Mexico [a]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	Origin Unknown																	
BA0003	Lebanon [a]	N	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+
BA0012	Argentina [a]	N	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-
BA0046	Argentina [a]	A	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
BA0052	Jamaica	A	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1032	M[j]	N	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-
BA1033	G28 (South Africa) [k]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1074	Penicillin Resistant [j]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BA1076	Penicillin Resistant [j]	N	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+

a. Centers for Disease Control, Atlanta, Georgia, USA
b. Dugway Proving Grounds, Utah, USA
c. Royal Veterinary College, London, England
d. Iowa State University, Ames, Iowa USA
e. Michigan Department of Public Health, E. Lansing, Michigan, USA
f. Arkansas Pountry and Livestock Diagnostic Laboratory, Springdale, Arkansas, USA
g. Textile Mill, Manchester, New Hampshire, USA
h. Veterinary Diagnostic Laboratory, Collage of Veterinary Medicine and Biomedical Sciences, Colorado State University, Ft. Collins, Colorado, USA
i. Center for Applied Microbiology and Research, Porton Down, Salisbury, England
J. United States Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Maryland, USA
k. Veterinary Research Institute, Department of Agricultural Technical Services, Rebublic of South Africa

NG = None Given; A = Aberrant; N = Normal

Table 2

Toxin Primers	Sequence
PA	5' GAC ACA TAC TAG TGA AGT ACA T
	5' AAT AAT CCC TCT GTT GAC G
LF	5' GAT GAA GCG CAG ATT CCT ATT GAG CCA
	5' GAT GAA GCA CTA AAT CCG CAC CTA GGG
EF	5' GTA TTA GAG TTA TAT GCC C
	5' TTC ATT CAA ACG ATC AAG C

Capsule Primers	Sequence
cap A	5' GGA ATT CAA GTT GTT GTC TCC ACT G
	5' AAG CAG AGT AAG ATG GAG TGG AAT T
cap B	5' CAT TCC TGT CCC TCC ACT TAA ATC AC
	5' TAG TAG ACA TAA TTA GGG AAG GAC GA
cap C	5' CGT CTC ATT CTA CCT CAC CTT AAA AT
	5' GAC TAG GTT TGT AAG GAC AGG G AG G

Hemolysin production

Although it is sometimes difficult to differentiate between *B. anthracis* and *B. cereus*, they can be distinguished by plating on both SBA and washed blood agar plates. *B. cereus* is hemolytic on SBA, whereas *B. anthracis* is not. However, *B. anthracis* does produce hemolysin on washed sheep blood agar plates[8]. All of the isolates in this study produced hemolysin on washed sheep blood agar.

Protective antigen and capsule production

PA production was detected by the appearance of an immunoprecipitin halo surrounding the colony after overnight capitalize incubation on immunoassay agar[9]. Of the 31 isolates tested, only four did not produce a halo on immunoassay agar. Two of these isolates, BA0019 and BA0046, did not contain the pX01 plasmid and therefore were not expected to produce a halo. However, pX01 plasmid DNA was isolated from BA0002 and BA0006 but PA production was not evident on the immunoassay agar. Capsule production was assayed by using bicarbonate agar and results were consistent with the presence of pX02, in all isolates, with one exception. Plasmid DNA could not be detected in isolate BA0046, although it produced capsule in air and in the presence of CO_2. As noted above, the capsule of BA0046 did not react with monoclonal antibody specific for poly-D-glutamic acid.

Penicillin sensitivity

B. anthracis is susceptible to penicillin, which is the drug of choice in the treatment of the disease[8]. All isolates were sensitive to 10 units of penicillin by the disc sensitivity method.

Sporulation

Spore formation in *B. anthracis* usually begins at the end of logarithmic growth and spores are visible as early as 24-48 h after incubation. The spores are oval and are central in position in the bacilli before their release[3]. Only three isolates, BA0001, BA0012 and BA0019, failed to produce spores on SBA.

Genetic analysis

Thirty-one isolates of *B. anthracis* were grown in broth cultures and screened for the presence of plasmid DNA. Twenty-three isolates contained both plasmids, six contained only pX01, and in two isolates, neither plasmid was detected. DNA samples were screened for the presence of toxin and capsule genes by using oligonucleotide primers prepared specifically to amplify these virulence determinants. Results of PCR analysis were compatible with the isolation of pX01 and pX02 with few exceptions. Plasmids the size of pX01 and pX02 were detected in BA1031, but oligonucleotide primers for PA, lethal factor (LF) and edema factor (EF) failed to amplify DNA from this isolate. Capsule genes were detected by PCR in this isolate. Of particular interest was isolate BA0046, in which plasmid DNA was not detected, but DNA was amplified, which was consistent with the presence of toxin and capsule genes.

Discussion

This study describes phenotypic, genetic, and biochemical characteristics of 31 isolates of *Bacillus anthracis*. The majority of the isolates exhibited characteristics typical of *B. anthracis*, although some variations were observed. The production of PA and capsule correlated, for the most part, with the presence of pX01 and pX02, respectively. The same was noted for the amplification of toxin and capsule gene DNA. Similar data have previously been described by Turnbull *et al*[10]. Variations observed seemed to be primarily associated with those isolates of animal origin.

South African isolate, BA1031, contains a plasmid of a size compatible with pX01, yet oligonucleotide primers specific for toxin genes failed to amplify target DNA. This could be attributed to a sequence variation, deletion or chromosomal integration of these genes. However, a halo was observed after overnight incubation on immunoassay agar, indicating the PA gene was present in this isolate. No additional assays were performed to further verify the presence of the remaining toxin encoding genes, or their products, LF and EF.

The only discrepancies found between genetic and phenotypic characteristics were in two isolates from Pakistan. A plasmid the size of pX01 was isolated from both BA0002 and BA0006. PCR analysis of these isolates yielded amplified products corresponding in size to the toxin gene DNA. Yet, these strains did not produce a halo on immunoassay agar. It is conceivable that these isolates have an alteration in the regulation of PA gene transcription, PA secretion or PA degradation.

The only human isolate to exhibit aberrant characteristics was BA0001. It exhibited colonies smaller in size than those of typical *B. anthracis* and failed to produce spores on SBA. In all other respects it appeared to be a typical strain.

Specifically of interest was BA0046, an isolate originating from Argentina. At times, it exhibited some unusual characteristics, such as the appearance of two colony types, suggesting that this isolate is not *B. anthracis*. The possibility of a contaminant was investigated, but could not be confirmed. The smooth colony type of BA0046 produced capsule, yet fluorescent monoclonal antibody stains specific to anthrax poly-D-glutamic acid capsule did not identify it as such. Plasmids could not be detected, but PCR analysis yielded amplified DNA products consistent with toxin and capsule genes. It is conceivable that these plasmid genes integrated into the chromosome.

Although a few penicillin-resistant isolates of *B. anthracis* have been reported[2,4,6], most are susceptible to the antibiotic. Isolates BA1074 and BA1076 were previously described as penicillin resistant, but were found to be sensitive by using disc sensitivity.

Isolates routinely used in our laboratory investigations include BA1002 (Vollum 1 B), BA1003 (Vollum), BA1004 (Ames), BA1008 (New Hampshire) and BA1043 (Sterne). The only notable difference among these strains is the lack of proteolytic activity of BA1003 (Vollum), which was previously described[11].

The data reported in this study have facilitated the establishment of a reference strain collection describing characteristics of animal and human isolates of *B. anthracis* of diverse geographical origins. Although most isolates were typical, some variations were noted that are most likely a reflection of strain diversity.

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Identification of *Bacillus anthracis* using the API 50CHB System

R. COGNE [1], N. E. STARES [2], M. N. JONES [3], J. E. BOWEN [3], P. C. B. TURNBULL [3] and J-M. BOEUFGRAS [1].

[1]bioMérieux S.A., La Balme Les Grottes, BP 2, 38390 Montalieu-Vercieu, FRANCE. [2]bioMérieux UK Ltd, Grafton House, Grafton Way, Basingstoke, Hampshire RG22 6HY, UK. [3]CAMR, Porton Down, Salisbury, Wiltshire SP4 OJG, UK.

Summary and introduction

The identification of *Bacillus anthracis* in specimens from animal products and the environment is often a challenge because of its similarity to *B. cereus* and *B. mycoides* which are frequently present in substantial numbers of such specimens.

The API 50CHB System (bioMérieux, France) is a micromethod allowing the identification of *Bacillus* species[2]. Although a previous publication[3] concluded that this method has the ability to distinguish *B. anthracis* correctly from the related species, some instances of erroneous identification of *B. anthracis* using this system have come to our attention. The purpose of this study was to address this problem.

Materials and methods

Biochemical tests

API 50CHB (bioMérieux, France) is a method allowing the acidification of 49 carbohydrates in microtubes. A suspension is made in the API 50CHB Medium with the microorganism to be tested, and each tube of the strip is inoculated. During the incubation, carbohydrates are fermented to acids which produce a decrease in the pH, detected by the colour change of the indicator.

For the identification of *Bacillus species*, API 50CHB is used in combination with the first 11 tests of API 20E and Nitrate reduction.

Procedure

API 50CHB and API 20E strips were inoculated following the manufacturer's recommendations, and incubated for 48 hours at 37°C. Each strain was tested by two different operators, from the same isolation plate. The suspensions used by the operators were coded in order that they did not know what they were testing.

Strains

58 *B. anthracis* representing 43 different isolation histories from various human, animal, environmental and geographical origins (see Table 1) and 36 strains belonging to other *Bacillus* species, were tested (see Table 2).

Table 1. Origin of the 58 *B. anthracis* strains

Origin	UK	Africa	Other/ Unknown
Human	3	3	3
Animal	3	13	7
Environmental	9	2	4
Other / unknown	3	1	7

Table 2. Strains belonging to species other than *B. anthracis*

Species	No.	Species	No.
B. cereus	5	*B. laterosporus*	1
B. licheniformis	5	*B. megaterium*	3
B. amyloliquefaciens	3	*B. alvei*	1
B. polymyxa	3	*B. macerans*	1
B. sphaericus	2	*B. brevis*	3
B. firmus	2	*B. pumilus*	2
B. subtilis	5		

Interpretation

The results were interpreted using the APILAB computerised identification system[1]. Two reference identification database versions were used; Version V. 2.1, the present commercialized version and Version X. 3.0, an experimental database, designed to prepare the next version to be marketed.

Results and discussion

Reproducibilty (see Table 3)

The percentage of agreement between the results obtained by both operators was calculated for each test. The agreement was good (greater than or equal to 95%) for 41 tests and 7 tests showed poorer reproducibility (less than 90% agreement).

Table 3. The agreement between two operators for the API 50CHB / API 20OE tests on 58 *B. anthracis* strains

API 50CHB			API 50CHB		
test n°	Substrate	%	test n°	Substrate	%
40	D TURanose	98.3	1	β-Galactosidase *	100.0
41	D LYXose	100.0	2	Arginine dehydrolase	96.6
42	D TAGatose	96.6	3	Lysine decarboxylase	98.3
43	D FUCose	100.0	4	Ornithine decarboxylase	98.3
44	L FUCose	98,3	5	Citrate	60.3
45	D ARobitoL	100.0	6	H2S	98.3
46	A ARabitoL	98.3	7	Urea	98,3
47	GluecoNaTe	94.8	8	Indole	96.3
48	2 Keto Glueconate	100.0	9	VP	100.0
49	5 Keto Glueconate	89.7	10	Gelatine	74.1
			11	Glucose	94.8
			12	Nitrate	91.4

Identification

The identification performance of both database (API 50CHB V. 2.1 and X. 3.0), on the profiles obtained for the 58 *B. anthracis* strains by both operators is summarised in Table 4. Among the 116 profiles obtained (2 per strain), 68 (58,6%) were misidentified using the API 50CHB V. 2.1 database. These profiles were misidentified as *B. cereus* (58), *B. mycoides* (2), *B. cereus* or *B. mycoides* (6), *B. megaterium* or *B. stearothermophilus* (1) and *B. pantothenticus* (1).

Using the experimental database API 50CHB X. 3.0, only 4 (3,4%) profiles were misidentified as *B. cereus* (1), *B. subtilis* (1), *B. licheniformis* (1), and *B. megaterium* or *B. firmus* (1). The identification performance of both databases on the 36 strains belonging to *Bacillus* species other than *B. anthracis* is shown in Table 5.

Table 3. The agreement between two operators for the API 50CHB / API 20E tests on 58 *B. anthracis* strains

test n°	Substrate	%
0	TEMOIN/ConTRoL	100.0
1	GLYcerol	58.6
2	ERYthritol	98.3
3	D ARAvinose	100.0
4	A ARAvinose	96.6
5	RIVose	98.3
6	S XYLose	98.3
7	L XYLose	100.0
8	ADOnitol	100.0
9	βMéthyl-D Xyloside	100.0
10	GALactose	98.3
11	GLUcose	100.0
12	FRUctose	91.4
13	MaNvoseE	89.7
14	SorBosE	100.0
15	RHAmnose	100.0
16	DULcitol	100.0
17	INOsitol	94.8
18	MANnitol	98.3
19	SORbitol	96.6
20	α-Méthyl-D Mannoside	100.0
21	α-Méthyl-D Glucoside	98.3
22	N Acetyl Glucosamine	100.0
23	AMYgdaline	98.3
24	ARButine	81.0
25	ESCuline	94.8
26	SALicine	74.1
27	CELiobiose	69.0
28	MALtose	100.0
29	LACtose	98.3
30	MELibiose	98.3
31	SACcharose / Sucrose	100.0
32	TREhalose	98,3
33	INUline	96.6
34	MeLeZitose	98.3
35	RAFfinose	100.0
36	AMiDon / Starch	79.3
37	GLycoGene	94.8
38	XyLiTol	100.0
39	GENtiobiose	93.10

API 50CHB identification database V. 2.1 (see Table 2) shows a poor performance for identifying *B. anthracis*. This is probably due to a limited number and the lack of representativity of the strains used in its initial development. The experimental version of the database X. 3.0 shows good results. The 4 misidentified strains were only misidentified by one operator. For 3 out of those 4 strains, the identification obtained by the other operator was correct. The lack of reproducibility between the 2 profiles for these 4 strains was due to differences in test reactions and these tests were among those which gave more reproducilble results for the other strains (Table 3). The performance of the experimental database version for identifying species other than *B. anthracis* is not decreased compared to the performance of version V. 2.1. It must be noted that no other *Bacillus* species was misidentified as *B. anthracis* using either of the two database versions.

Conclusion

The experimental version X. 3.0 of the API 50CHB database gives good results in this study for the identification of *B. anthracis*, and shows good promise as a suitable replacement for V. 2.1.

In the identification of *B. anthracis*, primary tests such as the lack of haemolysis and the lack of motility can be used to support the biochemical profile, in order to aid the differentiation between *B. anthracis* and *B. cereus*. This would reinforce further the reliability of the results obtained. The wide diversity of the strains used should result in a good performance in various situations. It would be interesting to confirm this prediction using profiles obtained from strains unrelated to the database.

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Table 4. Identification performance on 58 *B. anthracis* strains

	Operator 1				Operator 2			
	Correct Id.				Correct Id.			
	1 choice	> 1 choice	No Id.	Mis. Id.	1 choice	> 1 choice	No Id.	Mis. Id.
Data base V 2.1	0	0	26	32	0	0	22	36
Database X 3.0	28	27	1	2	25	26	5	2

Table 5. Identification performance on 36 strains belonging to species other than *B. anthracis*

	Operator 1				Operator 2			
	Correct Id.				Correct Id.			
	1 choice	> 1 choice	No Id.	Mis. Id.	1 choice	> 1 choice	No Id.	Mis. Id.
Data base V 2.1	3	2	23	8	11	2	18	5
Database X 3.0	18	9	6	3	14	8	6	8

Rapid method for the diagnosis of *Bacillus anthracis* infection in clinical samples using a hand-held assay.

J. BURANS, A. KELEHER, T. O'BRIEN, J. HAGER, A. PLUMMER and C. MORGAN.

Naval Medical Research Institute, Bethesda, MD, 20814-5607, USA

Summary

During the course of human and animal infections, *B. anthracis* produces a lethal toxin or an edema toxin, a component of which is protective antigen (PA). The concentration of PA in serum or blood increases over time to reach levels in excess of micrograms per ml. We have developed a rapid hand-held assay format which can detect PA in the blood, serum or other body fluids of infected humans or animals. The assay format is an immunochromatographic membrane based assay. The antigen capture assay utilizes a monoclonal capture antibody bound to a nitrocellulose membrane and a second monoclonal antibody of a different epitopic specificity bound to colloidal gold particles as a detection reagent. The assay requires ten minutes to perform and can detect as little as 25 ng/ml for the one step format. The one step chromatographic assay has been further evaluated against a panel of spiked and negative sera and was shown to have a specificity of 100 % at its target sensitivity of 25 ng/ml. This assay format was shown to be stable under room temperature storage conditions, extended and accelerated stability studies are ongoing. This rapid hand-held antigen capture assay was shown to be comparable in sensitivity to a 4 hour antigen capture ELISA using identical reagents.

Introduction

Bacillus anthracis, the etiologic agent of anthrax, causes an often fatal disease in domestic livestock, game animals and sometimes humans. The organism possesses two virulence factors, a poly-D-glutamic acid capsule and a three part toxin composed of protective antigen (PA), lethal factor (LF) and edema factor (EF). All virulent strains of *B. anthracis* produce capsule and the tripartite toxin.

PA, with a molecular weight of approximately 83,400 daltons, binds to specific cell receptors and is cleaved by a protease producing a 63,500 dalton cell bound receptor. The cleaved PA binds competitively with either EF or LF to produce active edema toxin or lethal toxin. Studies have shown that *B. anthracis* infection results in the release of PA into the blood. Thus the detection of PA in the blood of a febrile animal or human is diagnostic of *B. anthracis* infection. In this study we have developed a rapid hand-held assay format which can detect PA in serum, blood or other body fluids.

Materials and methods

Anti- PA Monoclonal Antibodies

Hybridoma cell lines designated 14-B7 and 3D-2[1] were used to produce the monoclonal antibodies (MAB) used in the PA immunochromatographic assay. The MABS were purified from mouse ascitic fluid by Protein A affinity chromatography. ELISA and Surface Plasmon Resonance (SPR) analysis were used to determine the epitopic specificity and affinity of the MABS for PA. MABS 14-B7 and 3D-2 were shown to be directed at two different epitopes on the PA molecule and that MAB 14-B7 was most suitable as a capture antibody and 3D-2 was most suitable as a detector antibody.

Protective Antigen (PA)

PA was generously supplied for this study by Dr P. Turnbull, Anthrax Section, Center for Applied Microbiology Research, Porton Down, UK.

Sera

Animal sera were obtained from Pel Freeze (USA) and human sera were obtained from Biospec (USA). All sera were stored frozen prior to use.

Immunochromatograpic assay production

The immunochromatographic assays were assembled as cards as follows (Figure 1). Lines of 14-B7 capture antibody were air brushed onto strips of nitrocellulose paper. Reaction control lines composed of goat anti-rabbit IgG were also sprayed onto the nitrocellulose paper. The membrane was then air dried and blocked briefly with polyvinylalcohol. Membrane strips with colloidal gold labeled 3D-2 detector antibody and colloidal gold labeled rabbit antibody were then mounted to overlap the blocked nitrocellulose membrane. A sample delivery pad composed of cotton lint paper was then applied to overlap the lyophilized colloidal gold membrane strip. Another cotton lint membrane pad was applied to overlap with the top of the nitrocellulose membrane. The assembled card was then cut into individual 7 mm strips which were then mounted into assay device holders (Figure 2).

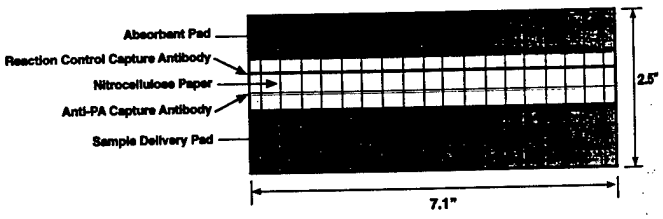

Figure 1. Immunochromatographic assay schematic

Figure 2. Immunochromatographic assay strip and device

Performing the immunochromatographic assay

Serum or body fluids are added directly to the sample well of the immunochromatographic assay device. The sample well of the device labeled "S" requires approximately 200 µl of fluid to fill. The fluid will then wick through the sample delivery pad, the lyophilized colloidal gold pad, the nitrocellulose membrane and then into the absorbent pad on the top of the assay strip. The assay is incubated for a total of 15 min at ambient temperature. The appearance of a red line in the test window of the assay marked "T" and in the reaction control window marked "C" indicates the presence of anthrax PA antigen at a concentration of at least 25 ng/ml of sample. The appearance of a red line only in the reaction control window "C" but not in the test window marked "T" indicates PA is not present in the sample or the concentration is below 25 ng/ml. The assay process is summarized in Figures 3 and 4.

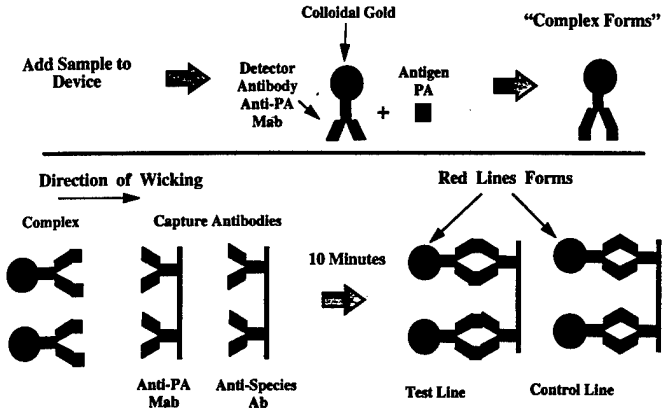

Figure 3. Anthrax protective antigen (PA) capture chromatographic assay

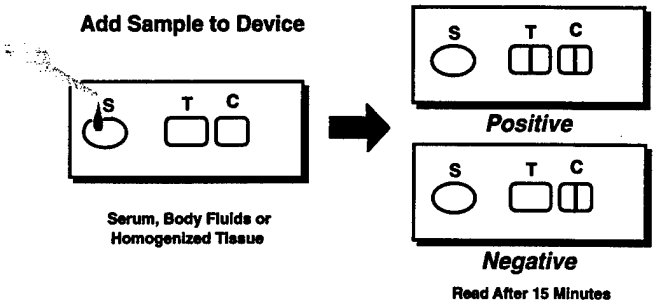

Figure 4. One-step hand-held assay. PA detection.

Results and discussion

Figure 5 is a photograph of a positive and negative immunochromatographic assay. The positive assay has red lines both in the "T" or test window and the reaction control "C" window. The negative assay has no line in the test window and a line in the reaction control window. The "one step" immunochromatographic assay was found to be easy to perform and to interpret. There was no requirement for additional equipment or reagents and personnel were easily trained to perform the assay.

The PA detection immunochromatographic assay was found to be both sensitive and specific for the rapid detection of PA in spiked human and animal sera specimens. The assay was compatible with human, goat, sheep and bovine sera. Sensitivity results for the PA detection immunochromatographic assay are summarized in Table 1. The assay was shown to have high specificity (>98%) for both negative human sera and diverse animal species which may become infected with anthrax such as sheep, goats, cattle and game animals such as zebra and wildebeest (Table 2). The assay was also tested for specificity by using culture fluids from related *Bacillus* species and sera spiked with unrelated proteins such as ricin and Staphylococcus enterotoxin B. In all cases the assay had 100% specificity. In a recent study conducted in the Etosha Game Park (P.M. Lindeque, J. P. Burans, P.C.B. Turnbull, manuscript in preparation), there was excellent correlation between detection of PA by the immunochromatographic assay, the suspicion of anthrax and confirmation by either smear, culture and or a combination of these two methodologies. In many instances where samples were obtained from animals which had just died from anthrax, the immunochromatographic assay was positive within 1 minute following addition of sample to the assay. This was due to the high concentration of PA in these samples.

The rapid PA immunochromatographic assay represents a new advance in the field diagnosis of anthrax in animals and humans. The assay is sensitive, specific as well as easy to manufacture, perform and interpret. The full potential, applicability and uses of this assay require additional field studies and evaluations which are currently being planned over the next 24 months.

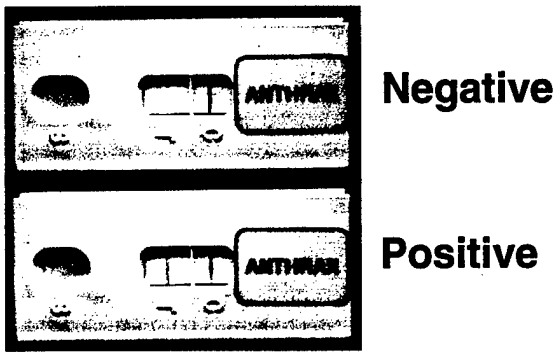

Figure 5. Immunochromatographic assay in use.

Table 1. Sensitivity of the anthrax protective antigen (PA) immunochromatographic assay in human serum

Concentration	Test results
100 µg[a]	4/4[b] (100%)[c]
100 µg	5/5 (100%)
1µg	5/5 (100%)
500 ng	5/5 (100%)
250 ng	5/5 (100%)
100g	5/5 (100%)
50 ng	5/5 (100%)
25 ng	5/5 (100%)
12.5 ng	3/5 (60%)
0	0/15 (0%)

a, concentration of PA per ml of human serum; b, ratio of positive assays to total number of assays performed; c, percentage positive

Table 2. Specificity of the PA immunochromatographic assay with human and animal sera.

Source of sera	No of sera tested	Result
Human	20	Negative
Zebra	2	Negative
Giraffe	2	Negative
Wildebeest	2	Negative
Springbok	2	Negative
Goat	1	Negative
Cattle	1	Negative
Sheep	1	Negative

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The taxonomic relationship between *B. anthracis* and the *B. cereus* group, investigated by DNA-DNA hybridization and DNA amplification fingerprinting (DAF)

HEIKE ROLOFF, PETRA GLÖCKNER, KATRIN MISTELE and R. BÖHM

Institüt für Umwelt- und Tierhygiene sowie Tiermedizin mit Tierklinik (460), Universität Hohenheim, Garbenstr. 30, D-70593 Stuttgart, Fed. Rep. of Germany

Summary and introduction

A two phase DNA-DNA hybridization method has been developed, using biotinylated total genomic DNA as probe and polystyrene-well microtiter plates as solid phase. Evaluation is done in a commercial ELISA-reader. The DAF is a PCR protocol with long (18 to 20 bases) non-random primers, which produce a band pattern in addition to the expected amplified product.

Using the hybridization assay and two different DAF protocols the *Bacillus cereus* group can be divided into three groups and several units of single strains behaving as a cluster. *B. mycoides* shows the widest separation from the other species of this group. The type strains of *B. cereus*, *B.thuringiensis*, and a *B. cereus* strain formerly called *B. medusa* belong to group I. Only few enterotoxin producing *B. cereus* strains have been found in group I and none in group III. Group II consists of several emetic strains of *B. cereus*, a diarrhea causing strain, several strains with unknown toxin status, and all strains of *B. anthracis*. The emetic strains of *B. cereus* (serotypes 1, 3, and 5) are closely related clusters. The fourth important cluster within group II consists of all strains of *B. anthracis*, regardless whether or not these strains possess the plasmids pX01 and pX02. Group 3 includes only a small number of strains of *B. cereus*.

Materials and Methods

35 strains of *B. anthracis*, 107 strains of *B. cereus*, 1 strain of *B. medusa* and the type strains of *B. mycoides*, *B. thuringiensis*, *B. coagulans*, *B. lentus*, *B. megaterium*, *B. subtilis*, and *B. firmus* were included in this study. Strains were provided by the DSM, Braunschweig, Germany, R.C.W. Berkeley, Bristol, U.K., A.-B. Kolstø, Oslo, Norway, and various culture collections.

DNA-DNA-hybridization

Single strand total genomic DNA was linked to the polystyrene plate. Six chemically biotinylated probes[8] were used and detection of the hybrids was done with the streptavidin-alkaline phosphatase system and nitrophenylphosphate as published earlier[5].

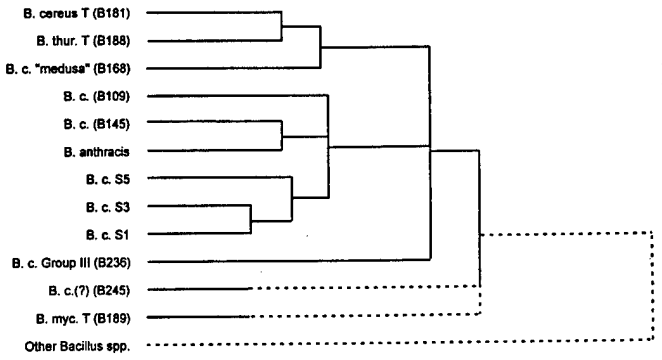
DNA amplification fingerprinting (DAF)

Sequence specific primers which produce species - or strain-specific band patterns were chosen. These patterns vary with the number and distance of annealing sites within the bacterial genome. The first protocol employed a primer pair of the *sec* Y gene of *B. subtilis*, SY 265 5'-GAG TGG TCT AAG CAA GGT-3' and SY 1120 5'-GGA AGA ATG GAA ATC ACG GC-3'. The assay was performed in 50 µl volumes each containing $MgCl_2$ 1.5 mM, KCl 50mM, Tris buffer, 10mM pH 9.0, primers 1 µM each, dNTPs 200 µM each, 2.5 U Taq polymerase, 100 ng template (1 µl), and sterile deionised water. For better reproducibility with large numbers of samples a hot start protocol employing AmpliWax beads was used.The cycling programme comprised firstly one cycle lasting 1 min at 94°C, 5 min at 20°C, and 4 min at 94°C, secondly 25 cycles lasting 1 min at 94°C, 90 sec at 43°C, and 90 sec at 73°C, and lastly 9 min at 72°C.

The second protocol using the M13 universal sequencing primer was as described previously[4]. The primer sequence was 5'-TAT GTA AAA CGA CGG CCA GT-3'. Purified DNA (100 ng) served as templates. The amplified DNA was visualized on agarose gels. The strains were grouped by visual comparison of the band patterns. The band size determination was carried out using bio1D software (Vilber Lourmat) on a personal computer.

Results

The hybridization results indicate the existence of at least three groups and several single strains behaving as a cluster among the strains in this study (Table 1). DAF provides a fast and reliable tool to indentify highly homogeneous clusters within the groups. 19 clusters and a number of single strains which behave as clusters could be identified by DAF. The taxonomic relationship which was obtained by combining the results of these two methods is demonstrated in Figure 1. The hybridization values of some clusters obtained with six different probes are shown in Table 1.



T = type strain, S = serotype, B. c. = *B. cereus*, B. myc. = *B. mycoides*, B. thur. = *B. thuringiensis*

Fig. 1: Taxononic relationship of selected members of the *B. cereus* group based on hybidization values and DAF

Group I comprises 8 clusters and several single strains. Cluster 1 contains the type strain of *B. cereus*. Cluster 3 includes the type strain of *B. thuringiensis*. *B. medusa*, is a single strain behaving as a cluster within group I. With the SY primers, the band patterns vary considerably between these clusters . With the M13 primer, however, all group I strains possess a bright band at 1020 bp which is not found in group 2 strains .

Group II comprises 10 clusters and several single strains behaving as clusters. Cluster 1 contains all serotype 1 strains of *B. cereus* which produce emetic toxin and some untyped strains of unknown toxin status. Cluster 2 consists of serotype 3 strains of *B. cereus* which have band patterns similar to the serotype 1 strains. Cluster 3 is formed by serotype 5 strains. Cluster 4 consists of all strains of *B. anthracis*. All strains have identical band patterns, regardless of the presence or absence of the plasmids pX01 and pX02.

Table. 1: Selected hybridization results

		PROBE					
		B181	**B168**	**B269**	**B109**	**A2**	**B236**
Group I	total	65-100%	28-47%	43-61%	41/64%	35%	41-55%
	B168	66%	100%	43%	41%	n.d.	41%
Group II	total	31-65%	20-26%	58-100%	55-100%	48-101%	48-53%
	B.cer. S 1	46-61%	20-23%	95-105%	57-73%	48-50%	49-50%
	B.cer. S 3	48-51%	20-21%	82-85%	65-68%	n.d.	n.d.
	B.cer. S 5	48-54%	21-25%	69-80%	64-74%	n.d.	n.d.
	B. anthr.	56-65%	22-25%	66-74%	66-75%	95-101%	n.d.
	B109 cluster	54%	24%	65-68%	95-100%	n.d.	53%
Group III	B236 cluster	50-55%	19-23%	49-56%	41-47%	n.d.	83-100%
	others	46-51%	18-23%	40-53%	37-48%	n.d.	45-52%
	B. myc.	38%	17%	39%	35%	27%	41%
Other Bacillus	10-15%	n.d.	7-11%	10-13%	7%	n.d.	

n.d: not done; B181: *B. cereus* type strain, B168: *B. cereus ("medusa")*, B269: *B. cereus* S1, B109: *B. cereus*, B236: *B. cereus*, A2: *B. anthracis*

Group III consists of only one homogeneous cluster. The remaining strains yielded how hybridization values with all probes used in this study and, therefore, cannot be assigned to a group. No similar fingerprints were observed among these strains. The type strain of *B. mycoides* had lower hybridization values than any other strain of the *B. cereus* group in this study.

B. cereus is a very heterogeneous species, not easy to distinguish from the closely related species *B. anthracis*, *B. thuringiensis*, *B. mycoides* and *B. medusa*.

B. mycoides is the most distinguishable species from the hybridization results. Nakamura and Jackson[6] (1995) had similar results.

Discussion

The results of this study show that *B. anthracis* is a small, highly homogeneous cluster within the *B. cereus* group. There is no difference between virulent and avirulent strains. API-tests lead to the same conclusion [2,3].

Some authors found that *B. anthracis* has a slightly higher degree of similarity with emetic *B. cereus* strains than with the type strain of *B. cereus*. Ash et al[1] found a 100% sequence homology in a section of the 16S rDNA between *B. anthracis* and the serotype 1 strain B269. The type strain of *B. cereus* differed in one nucleotide. Taylor and Gilbert[7] noted that *B. anthracis* and emetic *B. cereus* failed to ferment salicin whereas other *B. cereus* strains could ferment it. According to our results, the *B. anthracis* probe A2 yielded higher hybridization values with emetic *B. cereus* (~50%) than with the type strain and other group I strains (~35%).

There are many strains and clusters named *B. cereus* which have markedly lower hybridization values with the type strain of *B. cereus* than the type strains of *B. thuringiensis* and *B. anthracis* do. Considering these results the taxonomic status of the *B. cereus* group needs to be discussed again.

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Selective systems for the detection of *Bacillus anthracis* in environmental specimens

J.E. BOWEN, I. HENDERSON and P.C.B. TURNBULL

Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, UK

Summary

The maximum sensitivity of conventional methods for the isolation of *Bacillus anthracis* in environmental samples is approximately 5 spores per gram. However, if the spores are unevenly distributed, or present in very low numbers, they may remain undetected. There is consequently a requirement for improved detection methods for *B. anthracis* in environmental samples. Three approaches were attempted; development of a selective enrichment broth, use of differential germination prior to broth culture and biphasic aqueous/organic concentration. Biphasic separation, with enrichment when necessary, shows the most promise for further study into a selective system for *B. anthracis*.

1. Development of a selective enrichment broth for *Bacillus anthracis*.

Polymyxin, lysozyme, EDTA and thallous acetate agar (PLETA)[3] is the selective agar of choice in this laboratory for the detection of *B. anthracis* in environmental samples[8]. PLETA was systematically compared to other formulated selective agars, and a selective enrichment broth for *B. anthracis* developed.

Materials and methods

Agar media containing haematin and lysozyme[6], propamidine isethionate[4], PLET, phenylethanol[9] and TCB (trimethoprim, colistin and blood)[5] all reported to be selective for *B. anthracis*, were tested for growth of *B. anthracis* in naturally contaminated or seeded environmental samples. The active ingredients were also added to 10 ml volumes of brain heart infusion broth (BHIB), and 0.3 g of soil seeded with *B. anthracis* added. After 24 h incubation at 37°C growth of *B. anthracis* was looked for by subculture on blood agar (BA), and PLETA. PLETB (10 ml volumes), made with nutrient broth, heart infusion broth, or BHIB, and various concentrations of lysozyme, EDTA and thallous acetate (TA) was inoculated with a mix of 10^4 *B. anthracis* ASC 80 and 10^4 *B. cereus* F4433/73 spores. After 24 h incubation at 37°C, the resulting growth of *B. anthracis* was determined by subculture on BA. Optimum conditions were determined for pure culture. Seeded soil samples (0.3 g) were then tested and growth determined by subculture on PLETA.

The effects of various concentrations of mineral ions common in soil (Ca^{2+},Mg^{2+},Fe^{2+},K^+, Na^+,Mn^{2+},Al^{3+}) and different chelating agents (EDTA, EGTA, Chelex) on the growth of the mix of *B. anthracis* and *B. cereus* in 10 ml volumes of PLETB were tested, by addition to the broth and subsequent subculture on BA to determine the growth of *B. anthracis*.

B. anthracis was more readily isolated on PLETA than in PLETB, hence the merits of using semi solid[2] PLETA (0.1-0.9% agar) as a possible enrichment system were studied. Ten and 50 ml volumes of molten (50°C) agar were inoculated with the mix of *B. anthracis* and *B. cereus*, or with soil or plaster samples. The samples were vortexed and incubated overnight at 37°C. The growth of *B. anthracis* was determined by subculture on BA and PLETA.

Results and conclusions

PLETA was the most effective medium for selective growth of *B. anthracis* from the mix of *Bacillus* species naturally present in environmental samples, producing distinctive colonies of *B. anthracis* with greatly reduced background flora. The components of PLETA were also the most selectively effective in a broth system. BHIB was the best basal medium, and the chemical concentrations originally formulated by Knisely proved to be the optimal. EDTA, rather than TA, was essential for the selective growth of *B. anthracis*, but the combination of EDTA and TA gave the best selective growth (Fig.1). The addition of >2% (w/v) soil to PLETB prevented selective growth of *B. anthracis* (Fig. 2).

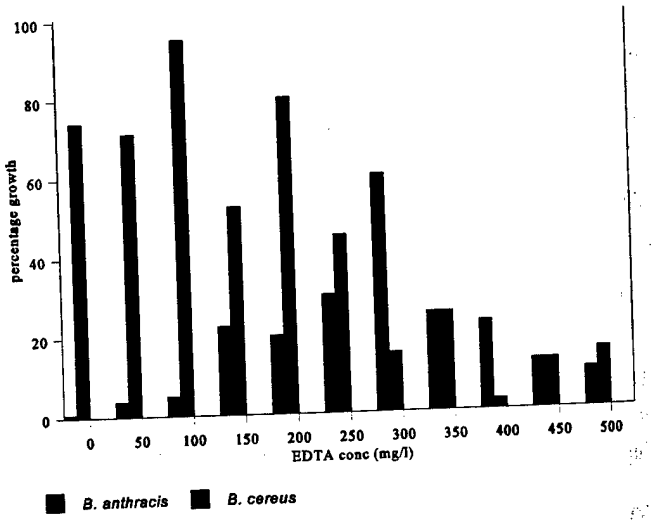

Fig. 1. The growth of *Bacillus anthracis* and *Bacillus cereus* in the presence of 40 mg/1 thallous acetate with increasing EDTA concentration.

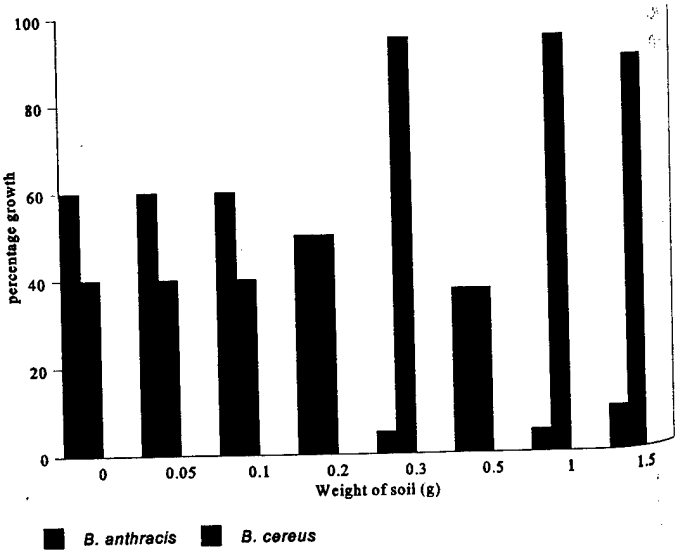

Fig. 2. The effect of the addition of soil on the selective growth of *Bacillus anthracis* in PLETB.

This inhibition of selective growth of *B. anthracis* was shown to be largely due to mineral ions present in soil chelating with the EDTA, thus preventing its synergistic action with TA. Different chelating agents removed excess of certain ions and returned the selectivity of PLETB. Examples of the results on which these conclusions are based are given in Table 1. Of the chelating agents tested (EDDA, EGTA, Chelex) EDTA was the most effective in PLETB.

Table 1. The effect on the selectivity of PLETB of addition of (i) EDTA (calcium chelating) with 0.0025M calcium chloride, (ii) Chelex (iron chelating) with 0.0025M iron chloride

	EDTA conc. mg/l	% Growth *B. anthracis*
PLET alone	300	95
PLET + $CaCl_2$	300	10
PLET + $CaCl_2$	500	70
PLET + $CaCl_2$	600	80
PLET + $CaCl_2$	700	90

	Chelex added	% Growth *B. anthracis*
PLET	0	95
PLET + $FeCl_2$	0	20
PLET + FeCl2	0.5g	75

The addition of 0.3 - 0.6% agar to PLETB increased the growth of *B. anthracis* to such a level that the sensitivity of detection of *B. anthracis* in environmental samples was comparable to that obtained with PLETA. However the system was liable to overgrowth of certain other naturally occurring *Bacillus* species. Further work is required to overcome this problem.

2. Germination of spores of *Bacillus anthracis.*

It seems possible that different germination requirements of *Bacillus* species[1] might allow selection of *B. anthracis* from a mixture of *Bacillus* species. In this study optimal germination conditions of *B. anthracis* and *B. cereus* were compared using a defined medium.

Materials and methods

Germination rates of spores of *B. anthracis* ASC 80 and *B. cereus* F4433/73, in different concentrations of L-alanine, inosine, guanosine and adenosine in SDW were determined. Heat shocked (62.5°C for 10 minutes) spores were added to 1 ml of germinant, and incubated at room temperature. Total viable counts and spore counts were done at 5 minute intervals on unheated and heated (62.5°C for 10 mins) samples.

Results and conclusions

Maximum germination of *B. anthracis* was obtained using 10 mM L-alanine and 1 mM inosine, or 10 mM L-alanine and 2mM adenosine (Fig. 3). The germination requirements for *B. anthracis* and *B. cereus* were the same, hence selection of one from the other on the basis of germination was not considered possible.

3. Biphasic separation

The high selectivity of the upper phase of an aqueous biphasic system[7] is reported to make it possible to concentrate spores directly from complex natural materials. This was tried using *B. anthracis* spores in environmental samples.

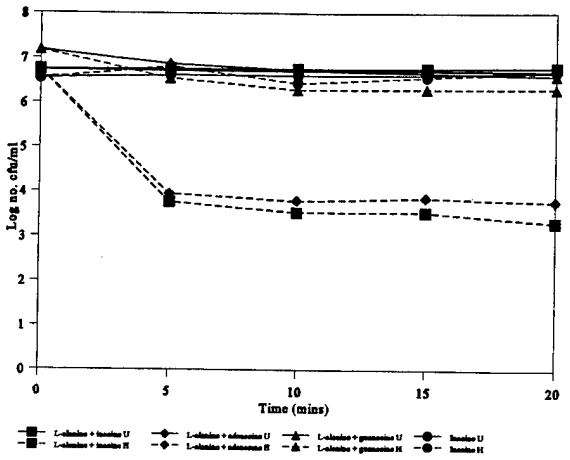

Fig. 3. Germination of spores of *Bacillus anthracis* and *Bacillus cereus* in different germination compounds.

Materials and methods

The following samples were tested: (i) naturally contaminated clay soil from the UK, (ii) sandy soil from a carcass site in Etosha National Park, Namibia, (iii) sterile UK soil seeded with *B. anthracis* ASC 245 spores and dried at 37°C for two weeks, and (iv) spores and sterile soil added separately. Potassium phosphate (2.9 ml of a 3.0 M solution, pH 7.0, polyethylene glycol (2.0 ml of 50% in SDW) and 3.7 ml SDW were combined with 0.1-0.5g soil. The samples were vortexed and counts done. After centrifugation (3000 rpm x 2 minutes), counts were again done on the top and bottom layers. For enrichment experiments, BHIB or PLETB was substituted for SDW with incubation at 37°C for 5 h.

Results and conclusions

With the sandy Etosha soil, a 5- to 10-fold concentration of spores in the top layer was observed. Conversely, with the UK soil, the majority of spores remained in the bottom phase, presumably attached to soil particles (Fig. 4). Separation occurred immediately from freshly seeded samples, but where the spores were dried onto soil they remained in the bottom layer. Enrichment led to some concentration of spores in the interphase from UK soil samples. PLETB led to detection of higher numbers of spores than BHIB (Fig. 5).

Further work will be carried out to determine how spores may be separated from soil particles prior to biphasic separation.

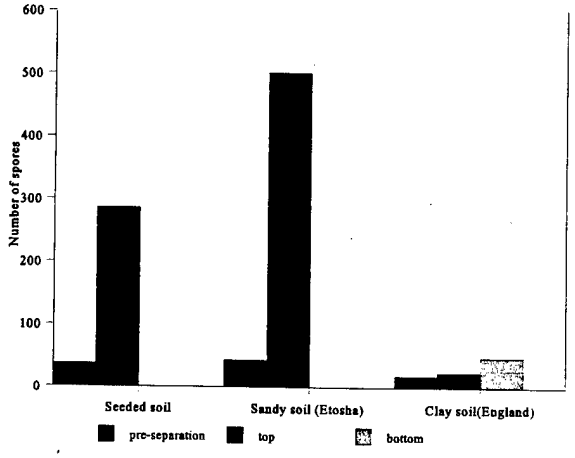

Fig 4. The concentration of spores of *Bacillus anthracis* in environmental samples using biphasic separation.

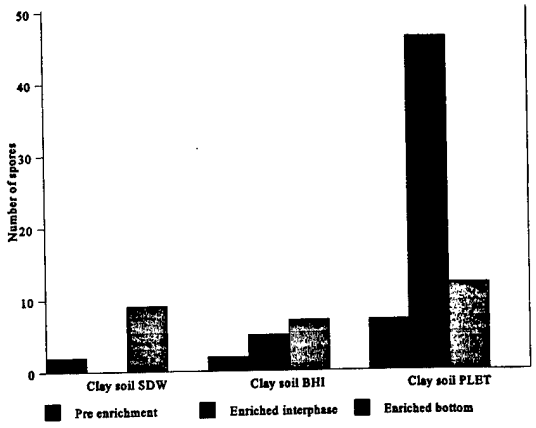

Fig 5. The effect of enrichment and biphasic separation on the concentration of spores of *Bacillus anthracis* in environmental samples.

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Encapsulation of *Bacillus anthracis* spores and spore identification

J. W. EZZELL AND T. G. ABSHIRE

Diagnostic Systems Division, U.S. Army Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md 21702-5011, USA.

Upon entry into a susceptible host, dormant *Bacillus anthracis* spores germinate and outgrow to become metabolically active vegetative cells. To protect itself during infection, the anthrax bacillus produces at least three virulence factors that inhibit various components of the host immune system. The lethal and edema toxins inhibit the function of macrophages and neutrophils, respectively, whereas the negatively charged poly-D-glutamic acid capsule inhibits phagocytosis.

As part of our study of *B. anthracis* infection in vivo and in simulations of in vivo conditions, we followed the transition of dormant spores into metabolically active cells in the presence of heat-inactivated horse serum[2]. Germination of *B. anthracis* spores in the presence of serum was analyzed by phase- and fluorescence microscopy after staining with fluorescein isothiocyanate (FITC)-monoclonal antibody conjugates (FA) to both the *B. anthracis* poly-D-glutamic acid capsule and the galactose/N-acetylglucosamine polysaccharide. The capsule monoclone (IgG) was designated as FDF-1B9 and the polysaccharide monoclone (IgM) was designated as EAII 6G6-2-3[1]. Most (>90%) spores within the population germinated but failed to outgrow in pure serum for periods up to 24h. One third of the spores that germinated in serum not only became swollen, dark and nonrefractive under phase microscopy, but also became encapsulated. Capsule, visualized by FA, appeared to emerge from the spores in the form of blebs (Figure 1), which enlarged with time and often coalesced to form a continuous layer (Figure 2, schematics 4 to 6). In contrast, virtually all the spores (95 to 99%) germinated and formed capsules when incubated for 30 to 45 min in heart infusion broth fortified with a mixture of amino acids, adenine, uracil, salts, 0.8% sodium bicarbonate and 50% heat-inactivated horse serum. The spores not only germinated but outgrew to form fully encapsulated vegetative cells within 1 to 2h in this medium. However, spores incubated 30 to 45 min in fortified heart infusion broth without serum and sodium bicarbonate did not form capsules, and only the emerging vegetative cells were stained by FA to anthrax polysaccharide during outgrowth (Figure 2, schematics 2 and 3).

Based on our findings, we developed methods for identifying *B. anthracis* spores which involved a short incubation of spores in two media and staining with fluorescein isothiocyanate/monoclonal antibody conjugates to the capsular poly-D-glutamic acid and the cell-wall associated galactose/N-acetylglucosamine polysaccharide. Although other *Bacillus* species produce polyglutamic acid capsular material (i.e., certain *B. subtilis* and *B. licheniformis* strains), and two *B. cereus* strains produce the galactose/N-acetylglucosamine polysaccharide[1], the combination of both traits is strongly indicative of *B. anthracis*. The specificity of the polysaccharide for *B. anthracis* has been demonstrated[1,2].

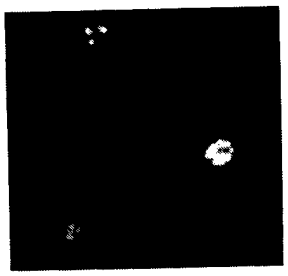

Figure 1. Encapsulation patterns of three *B. anthracis* spores after 30 min incubation in fortified beef heart infusion supplemented with 50% heat-inactivated horse serum and 0.8% sodium bicarbonate and staining with FDF - IB9 Mab - FITC conjugate. Spores viewed by epifluorescence microscopy on a nucleopore filter.

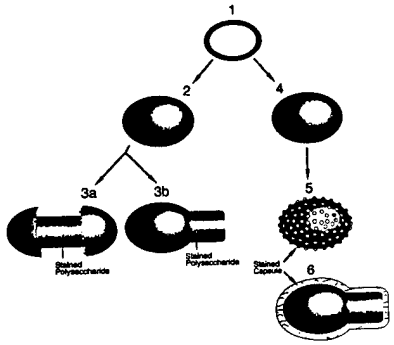

Figure 2. Diagrammatic representation of *Bacillus anthracis* spore germination and outgrowth. Events (1, 2, 3a and 3b) depicted on left side represent incubation in fortified beef heart infusion and staining with EAII 6G6 monoclonal antibody - FITC conjugate. Events (1, 4, 5 and 6) depicted on the right side represent incubation in fortified beef heart infusion supplemented with 50% heat-inactivated horse serum and 0.8% sodium bicarbonate and staining with FDF-1B9 monoclonal antibody-FITC conjugate.

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Plasmid and protein profiles of *Bacillus anthracis* strains isolated in China

LIANG XUDONG, MA FENGGING, YU DONGZHENG and LIN TAO

Institute of Epidemiology and Microbiology, Chinese academy of prevention medicine, P.O. Box 5, Changping 102206, China

Summary

The strains of *Bacillus anthracis* isolated from different parts of China were analysed for their plasmid spectrum and their whole cell protein SDS-PAGE patterns. All the virulent strains carried two plasmids, with molecular weights the same as those reported for pXO1 and pXO2. Several avirulent strains isolated from the feces or vomitus of the patients carried only one plasmid. This plasmid was identified as pXO2 by the PCR and probe hybridization analysis. It suggested that their attenuation was caused by the loss of the toxin genes. There were significant differences between the SDS-PAGE patterns of the virulent, avirulent and vaccine strains. The patterns had high diversity and could be divided into different modes. They could provide the clues is to the source of anthrax transmission.

Introduction

Anthrax is a zoonosis with heavy risk to human beings. Intensive research has been done on its causative agent, *Bacillus anthracis*. It was identified by Koch and Pasteur in 1876. In the 1930's, Sterne successfully developed an effective veterinary vaccine still used extensively. The appearance of antibiotics provided a strong measure in anthrax control. Since the 1980's, research has had a series breakthroughs in the field of its virulence determinants, pathogenic mechanisms, and vaccine development, mainly due to the development of molecular biological techniques. To date, however, little is known about the molecular epidemiological aspect of anthrax. In this paper, research on the plasmid and protein profiles of *B. anthracis* strains is reported, and their epidemiological significance discussed.

Materials and methods

Strains

Bacillus anthracis strains used in this research are listed in Table 1. Twelve of these were representative strains from those isolated during surveillance in different areas in this country, and the other 10 were selected from the store strains in the *Bacillus anthracis* Laboratory, Chinese National Center for Medical Bacteriology, kindly provided by Dr. Dong Shulin.

Plasmid analysis

Plasmid DNA preparation was basically done by the method of Kato-Liu[1,2], with little variance. Briefly, bacteria were incubated in Luria Broth for 8 hours at 37°C. The cultures were transferred to fresh medium and incubated at 37°C overnight, after which the cells were harvested. After lysis, DNA was extracted 3 times by phenol-chloroform, precipitated by ethanol, and resuspended in TE. Electrophoresis was run in 0.5% agarose gel for 6-8 hours at 80v. Stained by ethidium bromide, the photos were taken on ultraviolent lamp.

SDS-PAGE

Bacteria were incubated in Luria Broth for 16-18 hours at 37°C, and were killed by adding chloroform or peroxyacetic acid to the suspension to a concentration of 10%. 24 hours later, after sterility had been confirmed, the cells were spun down at 12000 rpm for 20 min. Sediments were washed 3 times in 0.02M PBS (pH 7.4), then resuspended in physiological saline. The contents of whole cell protein was determined according to Bradford[3]. The samples were split into aliquots, and kept at -25°C until required.

The gels were prepared according to Laemmli[4], Electrophorisis was performed on vertical gels, with 12% running gel and 5% stacking gel. Each sample (25 microlitre) was boiled for 10 minutes, then loaded with an equal volume of 2x loading buffer (10% mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol in 100 mM Tris-Cl, pH6.8). The terminal concentration of protein was 1 mg/ml. Electrophoresis was performed at a steady current of 10 mA for 16 hours. The gels were stained in Coomassie brilliant blue for 4 hours, and photographs were taken after destaining.

Table 1: Data on the *Bacillus anthracis* strains used in this study

Virulent strains			Avirulent strains		
Strain code	**Origin**	**From**	**Strain code**	**Origin**	**From**
Xizang3	Human	Tibet	Xizang10	Human	Tibet
Xizang5	Cattle	Tibet	Sichuan10	Human	Sichuan
91043	Soil	Xinjiang	Guangxill	Human	Guangxi
91004	Sheep	Xinjiang	91013	Human	Xinjiang
92002	Fur	Xinjiang	Guangxi10	Human	Guangxi
Xin-Epi	Marmot	Xinjiang	Pasteur I*	Vaccine	
Jiangxil*	Human	Jiangxi	Pasteur II*	Vaccine	
Eip-6*	Cattle	Inner Mongolia	CTU-1*	Vaccine	
A04*	Human	Hebei	Sterne*	Vaccine	
63504-1*		Out of China	A16R*	Vaccine	

* Provided by Dr. Dong Shulin

Results

Plasmid species

In all the virulent strains, two species of plasmid, PXO1 and pXO2, with the molecular weight of 110MD and 60MD respectively, could be detected as shown in Figure 1. In the avirulent strains, only one species of plasmid could be detected. The strains of 91013, Xizang10, Guangxill, and Sichuan10 could not form a capsule, but they carried pXO2 instead of pXO1, shown in Figure 2. This plasmid has been verified as pXO2 by PCR and specific probe.

Whole cell protein profile

More than 30 main bands could be distinguished in the whole cell protein profile, as shown in Figure 3 and Figure 4. There were apparent differences between the virulent, the avirulent, and the vaccine strains. With those strains, the bands with molecular weights of 86, 62, 48, 41, 28, 24, and 20 kD seem to differ significantly. Most of the strains had the 86, 28, and 24 kDa bands but avirulent and vaccine strains frequently had different protein profiles, as shown in Table 2.

Table 2: The main protein profiles of the different *Bacillus anthracis* strains

Protein bands (kDa)	86	62	48	41	28	24	20
Most of the strains	+	-	-	-	+	+	-
Jiangxil	+	-	-	-	-	+	-
Guangxi10	+	-	-	+	-	+	+
Guangxill	+	+	-	-	+	+	-
Sichuanl0	+	-	-	-	+	-	-
91013	+	-	+	+	+	+	+
Pasteur II	-	-	+	-	-	-	+
Al6R & Xizangl0	+	-	+	-	-	-	-

Discussion

It is thought that, in evolutionary terms, *Bacillus anthracis* is a young species. All strains isolated in nature are very alike; no apparent sub-types occur within the species. This is true in our researches; all the virulent strains isolated in the surveillance in different parts of China carried the same plasmids and had similar protein profile.

On the other hand, it has long been noticed that some attenuated strains of *B. anthracis* existed in the nature, and sometimes it is difficult to distinguish them from *B. cereus* strains by routine methods. In practice, they were frequently dismissed as "anthracis-like" strains, almost no one bothering to keep and study them. In our recent surveillance, besides a great number of the virulent strains, several noncapsular avirulent strains were isolated, which we have studied from both the plasmid and protein profile aspects.

The plasmid analysis showed that all the virulent strains carried pXO1 and pXO2 plasmids. There is no molecular weight variation between the strains isolated in different areas. All the avirulent strains isolated in the recent year-long surveillance were pXO1-/pXO2+. This is quite different from another report[5]. The PCR and probing verified that this plasmid irrefutably was pXO2. When these strains were isolated, the morphological study showed that they were noncapsular; we have still not ascertained the reason. PCR and probing verified that they truly lost their toxin genes, so their attenuation should be caused by the loss of the toxin plasmid. Interestingly, all these strains were isolated from vomitus or feces of patients. The epidemiological significance of this has yet to be determined.

SDS-PAGE analysis showed that there were apparent differences between the profiles of the virulent, the avirulent and the vaccine strains. The virulent strains have similar protein profile, with three main bands of 86, 28, and 24kD, except Jiangxil, which had lost the 28 kDa band. As well as the loss of one of these bands, avirulent strains had different protein profiles. CTU-1 and Sterne vaccine strains had similar profiles to the virulent strains, suggesting they possessed the most complete protective antigens. The profile of Al6R was similar to that of the Xizang10, suggesting that they could be from a common ancestor. Protein profiles are stable characters. Further study on this could provide a method to trace the anthrax transmission relationships.

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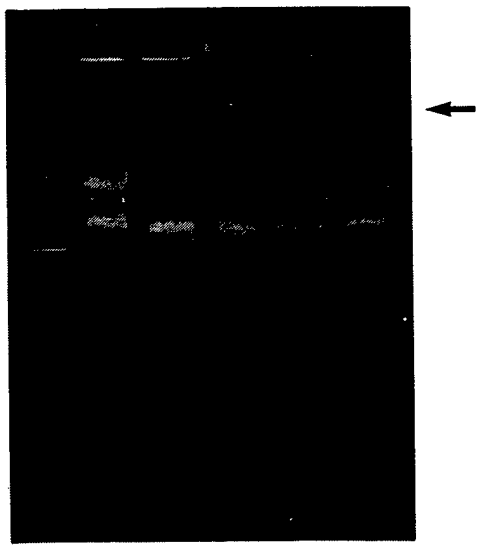

Figure 1: Plasmids carried by the strains of *B. anthracis*

Lanes: 1, Molecular markers
2, 63504
3, Xizang3
4, 92002
5, AO4
6, Jiangxil

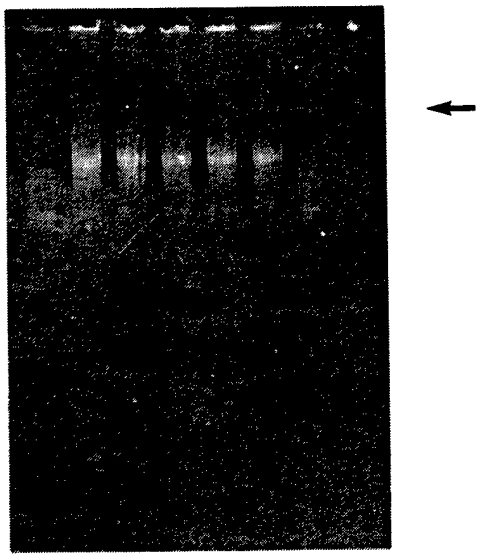

Figure 2: Plasmids carried by the strains of *B. anthracis*

Lanes: 1, 95043
2, 91013
3, Xizang10
4, Guangxill
5, Sichuan10
6, Pasteur II

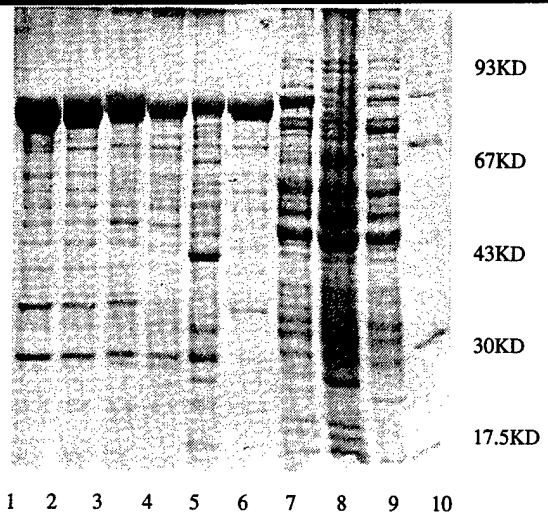

Figure 3: Protein profiles of the strains of *B. anthracis*

Lanes: 1, Xizang3
2, Epi6
3, 63504-1
4, Jiangxil
5, Guangxi10
6, Sichuan10
7, Xizang10
8, Pasteur II
9, Al6R
10, Molecular markers

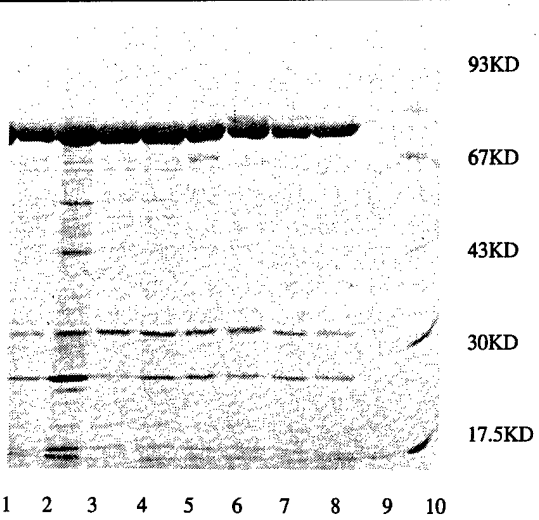

Figure 4: Protein profiles of the strains of *B. anthracis*

Lanes: 1, A04
2, 91013
3, 91004
4, 91043
5, 92002
6, Xin-Epi
7, Xizang5
8, CTU-1
9, Sterne
10, Molecular markers

Specific oligonucleotide primers for rapid identification of *Bacillus anthracis* strains

G. PATRA[1,2], P. SYLVESTRE [2], V. RAMISSE [2], J. THERASSE [2] and J.-L. GUESDON [1].

[1] *Laboratoire de Prédéveloppement des Sondes, Institut Pasteur, 75724 Paris Cedex 15, France, and*
[2] *Laboratoire des Biotechnologies, Centre d'Etudes du Bouchet, B.P. N°3, 91710 Vert-le-Petit, France*

Summary and introduction

Identification of *Bacillus anthracis* by PCR assay has been widely developed on the basis of the well-known virulence factors *lef, cya, pag* and *cap* localized on plasmids pXO1 and pXO2 respectively[1]. With the aim of identifying a marker specific for *B. anthracis* chromosome, we have constructed a cosmid library from a pXO1$^-$/pXO2$^-$ strain of *B. anthracis*. By screening this library with an appropriate Southern-blot technique, we identified a sequence specific (named Ba813) for *B. anthracis* chromosome which is apparently missing in other genus or *Bacillus* species.

A PCR-based test was developed for the detection of *Bacillus anthracis*. One pair of oligonucleotide primers, R1 and R2, was designed to amplify a 152-bp fragment from the Ba813 sequence. The amplified product was analysed by non-radioactive sandwich hybridisation in microtiter plates using two oligonucleotides. The capture oligonucleotide C1 was covalently linked onto aminated wells of microtiter plates. The detection oligonucleotide D3 was labelled with biotine. The hybrid molecules were detected by avidine conjugated with alkaline phosphatase and chromogenic substrate. The test requires only a thermal cycler and a conventional microtiter reader, moreover it can be readily done on a large scale[2].

Materials and methods

Strains

The six strains of *B. anthracis*, 7700, 7702, 4229, 6602, Davis TE 702 and Cepanzo, were kindly provided by Dr M. Mock. The 20 other *B. anthracis* strains were obtained from various French culture collections. Eleven species of *Bacillus* and sixteen heterologous bacteria representing twelve genera were analysed by DNA hybridisation assay for the specificity evaluation.

Construction of a Bacillus anthracis cosmid library

Genomic DNA from *Bacillus anthracis* 7700 was partially digested with *Hin*dIII, and the 30- to 40-kb DNA fragments were cloned in pHC79 cosmid. The recombinant cosmids were packaged and used to infect *Escherichia coli* HB101.

Library screening and isolation of a fragment hybridising specifically to B. anthracis DNA

Cosmid DNA samples were digested with restriction enzyme *Hin*dIII, electrophoresed through a 0.7% agarose gel, and transferred onto filters for Southern blot hybridisation analysis. Membranes were successively probed with *B. anthracis* 7700 and *B. cereus* DNA labelled with [α-^{32}P]dCTP by using a random priming method. A 2.1-kbp *Hin*dIII fragment (named A6F8) which strongly hybridised with *B. anthracis* was eluted from the agarose gel, purified, and the specificity of A6F8 fragment was tested by hybridisation with DNA isolated from closely related *Bacillus*. The *Hin*dIII - *Eco* RI extremity of A6F8 fragment was subcloned into pUC18; the resulting recombinant plasmid was named pBa813. The sequence of the insert was determined by the chain termination method of Sanger[4].

PCR experiments

The following oligonucleotides derived from the Ba813 sequence were used as the primers in the PCR experiments : R1 (5'-TTAATTCACTTGCAACTGATGGG-3') and R2 (5'-AACGATAGCTCCTACATTTGGAG -3'). PCR tests were performed with the Pharmacia LKB-Gene ATAQ Controller and under the following conditions: total volume (50 µl), oligonucleotides (0.5 µM), dNTPs (200 µM), $MgCl_2$ (1.5 mM), Taq-DNA polymerase AmpliTaq® Cetus (1U). Total genomic DNA (~ 100 ng) was denatured (5 min at 94°C) and submitted to 40 PCR cycles : 1 min at 94°C, 1 min at 60°C, 1 min at 72°C). The specificity of R1R2 products was tested by Southern Blot hybridisation assay with radioactive probe and by sandwich hybridisation assay.

Southern Blot analysis

The pBa813 plasmid was labelled with [α-32P]dCTP by using a random priming method and used as the probe.

Non-radioactive hybridisation

The sandwich hybridisation assay on CovaLink® NH strips was performed following the protocol already published[2] and using the capture probe
C1 (5'-GCCAGGTTCTATACCGTATCAGCAA-3') and the detection probe
D3 (5'-TTTGAAGCATTAACGAGTTACTC-3') selected within the R1R2 sequence.

Results and discussion

The cosmid library constructed was representative of the complete *B. anthracis* genome and contains a set of 600 recombinant clones. The clones were analyzed by Southern blotting by using *B. anthracis* 7700 and *B. cereus* total DNA as probes. One clone was found to contain a 2.1-kb fragment that hybridised strongly with *B. anthracis* DNA but not with *B. cereus* DNA. An extremity of the fragment (Ba813) was subcloned and sequenced. A search in EMBL and Genbank data bases did not reveal significant homologies with sequences previously described.

Oligonucleotides R1 and R2 derived from Ba813 sequence were used as primers in PCR assay. The amplification specificity of the R1R2 sequence was tested with different DNAs extracted from various *Bacillus* species and non-*Bacillus* bacteria. By using these primers, amplification of the expected 152-bp fragment was observed for the strains belonging to the *B. anthracis* species.

The identity of the 152-bp PCR product was verified by Southern blot analysis and sandwich hybridisation.

For Southern blot analysis, PCR products were probed with the pBa813 plasmid. A specific hybridisation signal was observed with DNA extracted from *B. anthracis* 7700 ($pXO1^-/pXO2^-$); *B. anthracis* 7702, Cepanzo and 957 ($pXO1^+/pXO2^-$); *B. anthracis* 4229, 6602, Davis TE 702 ($pXO1^-/pXO2^+$); *B. anthracis* 955, 645, 552, 470, 170, 657, 516, 300, 554, 779, 832, 204, 663, 376, 846, 256, 893, 582, 282 ($pXO1^+/pXO2^+$) while no signal was obtained with DNA extracted from the 11 closely related *Bacillus* and the 16 heterologous bacteria.

In addition the PCR products were submitted to non radioactive sandwich hybridisation test. The results obtained were in agreement with those obtained by Southern blot analysis, indeed significant OD were observed only with *B. anthracis* DNA (Figure 1).

The described combination of microplate sandwich hybridisation and PCR seems to be a suitable method for rapid detection of *Bacillus anthracis*. When required, the potential virulent or "avirulent" character can be specified using the Multiplex PCR assay described elsewhere[3].

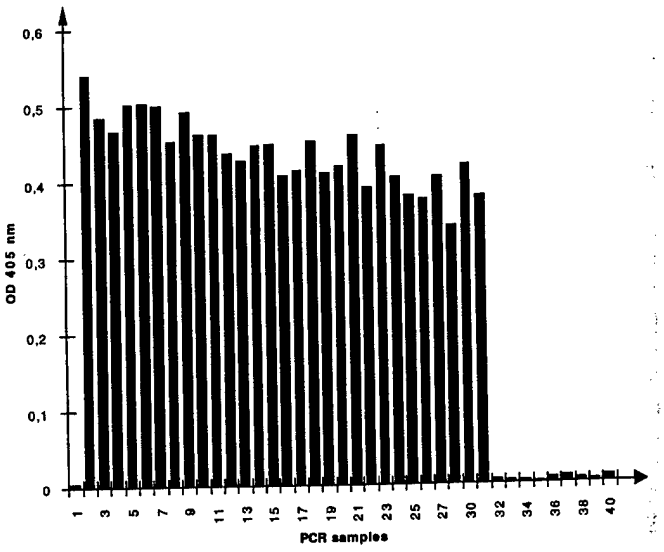

Figure 1: Sandwich hybridisation results
Samples were : without DNA (1), *B. anthracis* strains 7700, 7702, Cepanzo, 957, 4229, 6602, TE 702 Davis, 955, 645, 552, 470, 170, 657, 516, 300, 554, 779, 832, 204, 663, 376, 846, 256, 893, 582, 282 (2 to 31), *Bacillus cereus* (32 and 33), *Bacillus thuringiensis* (34 and 35), *Bacillus subtilis* (36), *Bacillus pumilus* (37), *Bacillus megaterium* (38), Heterologous PCR products (39 and 40).

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A nested PCR and DNA-amplification-fingerprinting method for detection and identification of *Bacillus anthracis* in soil samples from former tanneries

W. BEYER*, P. GLÖCKNER, J. OTTO and R. BÖHM

University of Hohenheim, Institute of Environmental and Animal Hygiene (460), Garbenstraße 30, 70599 Stuttgart, Germany

Introduction

While the detection of *Bacillus anthracis* in clinical specimens generally does not cause any problem, the isolation and identification of anthrax spores in environmental samples is comparatively difficult. This is mainly due to the lack of effective enrichment procedures, the presence of high numbers of other aerobic spore-forming bacteria which may easily overgrow the pathogens and the presence of chemical compounds inhibiting the germination of the relevant spores or interfering with the detection procedures[1,13]. Several methods had been described in the literature for improving selective culture media[5], to increase the sensitivity of techniques based on labelled antibodies[1,10,13], and to introduce hybridization techniques for the diagnosis of *Bacillus anthracis*[9], but generally the detection limit was found to be 10^3 spores/g and several limitations resulting from the nature of environmental samples have been reported[10,12,13].

Recently some successful attempts were made to detect a single *B. anthracis* spore by PCR[2,4,6,11]. While most of these experiments were conducted with DNA from pure bacterial cultures, we focused our efforts on the detection of *B. anthracis* in native environmental samples, such as soil and waste from former tannery sites.

The virulence of *B. anthracis* depends on the presence of both plasmids: pX01, which encodes for the lethal factor, the protective antigen, and the edema factor[8] and pX02, which encodes for the capsule[3]. A method which is designed to detect virulent *B. anthracis* should therefore be able to show the presence of both plasmids.

Materials and methods

Table 2. Primers used in the nested PCR and in the DNA-Amplification-Fingerprint

Primer[a]	Sequence (5'-3')	Location within genes[b]	Predicted size of amplified product
CAP6	TACTGACGAGGAGCAACCGA	506-525	
CAP103	GGCTCAGTGTAACTCCTAAT	41-1522	1035 bp
CAP9	ATGTATGGCAGTTCAACCCG	617-636	
CAP102	ACCCACTCCATATACAATCC	1394-1375	777 bp
PA8	GAGGTAGAAGGATATACGGT	2452-2471	
PA5	TCCTAACACTAACGAAGTCG	3048-3029	596 bp
PA7	ATCACCAGAGGCAAGACACCC	2631-2651	
PA6	ACCAATATCAAAGAACGACGC	2841-2621	210 bp
SY265	GAGTGGTCTAAGCAAGGT		
SY1120	GGAAGAATGGAAATCACGGC		855 bp

a The primers CAP6/103 and PA8/5 were used in the first PCR stages, the primers CAP9/102 and PA7/6 were used in the nested PCR stages, primers SY265/1120 are from the sequence of the secY gene of *B. subtilis*.

b The position numbers are those in the published nucleotide sequences of genes B and C within the cap region of pXO2 (Makino et al., 1989) and of the PA gene of pXO1 (Welkos et al., 1988).

Soil characterization

The soil samples used in this study were collected from the location of a former tannery. They consisted of clay material (particle size 0.002-0.06 mm) or sandy to gritty material (particle size 0.06-2.0 mm). There were big variations in the organic compounds ranging from humous to mudded and peaty materials. The maximum total organic carbon content was 250.4 mg/g, the pH value ranged from 4.6 up to 12.5. The soil samples were contaminated with animal hairs, leather waste, and cinders. The analysis of inorganic compounds yielded chromium (84.5 mg/g), arsenic (3.5 mg/g), copper (3 mg/g), lead (1.7 mg/g), zinc (1.3 mg/g), iron (43.6 mg/g), magnesium (18.1 mg/g), calcium (436,8 mg/g), mercury (13 µg/g), cadmium (250 µg/g), manganese (582 µg/g) and sulfides (3.3 µg/g). The content of naphtalene was 2.8 mg/g at maximum.

Preparation of soil material and nonselective enrichment of bacteria

From the location of a former tannery 100 g of the original soil material was seeded with serial 10-fold dilutions of *B. anthracis* spores of the strains A73 or A15 from 4 x 104 down to 4 x 100 spores. The spiked samples were then incubated with 200 ml deionized water and 60 g of glass beads at room temperature with constant shaking at 150 rpm for 15 h. The next day the samples were filtered successively through stainless steel sieves of 500 µm and 250 µm mesh. The filtrate was centrifuged for 25 min at 5000 x g at 4 °C and the pellet resuspended in 100 ml TSB (Trypticese soy broth, Oxoid). The suspension was incubated overnight at 37 °C with constant shaking at 150 rpm. The next day 100 µl of the enrichment broth was inoculated in 10 ml of fresh TSB and incubated at 37 °C for 6 h to avoid spore formation. To this culture, H_2O_2 was added to a final concentration of 3 % and the sample further incubated at 37 °C for 1h to kill vegetative cells. Then it was centrifuged for 20 min at 3000 x g at 4 °C and the pellet was washed twice with 10 ml of buffer containing 0.05 M Tris-HCl, pH 7.2; 0.15 M NaCl. From the second resuspension 1 ml was spread on blood agar and the plates incubated over night at 37 °C to check the sterility of the sample. The remainder of the sample was kept frozen at -20 °C until the DNA preparation.

DNA preparation

The samples were thawed at 37 °C and centrifuged for 20 min at 3000 x g at 4 °C. The pellet was resuspended in 0.5 ml TE (Tris-HCl, 10 mM, pH 8.0; Na2EDTA, 1 mM) and after the addition of 30 µg/ml mutanolysin (Sigma) and 5 mg /ml lysozyme (Serva) it was incubated at 37 °C for 1 h. After addition of proteinase K (200 µg/ml, Serva) and SDS (1 % final concentration), the bacteria were lysed at 50 °C for 30 min. Then TE buffer, sodium chloride, and SDS were added to a final volume of 3ml, containing 1 M final concentration of NaCl and 1 % final concentration of SDS. The mixture was kept refrigerated (4 °C) for 45-60 min. The bacterial debris was then pelleted for 15 min at 13000 x g at 4 °C. One volume of equilibrated phenol was added to the supernatant and the mixture was emulsified by agitation; subsequently the same volume of chloroform was added and the mixture emulsified again. The emulsion was centrifuged for 20 min at 3000 x g at 4 °C. The aqueous supernatant was transferred to a fresh tube and subjected to extraction once with 1 volume of chloroform. The upper aqueous phase was transferred to a fresh tube and

incubated with RNase A (200 µg/ml) at 37 °C for 15 min. The DNA was precipitated by the addition of 2.5 volumes of ice-cold 96 % ethanol and then spooled on a glass rod. In the event of the development of a floccy precipitate, the DNA was pelleted at 13000 x g for 5 min. The DNA was washed with 70 % ethanol, dried in air and then resuspended in 100 µl TE (pH 8.0). The consistency, concentrationand purity of the DNA was evaluated by gel electrophoresis in an 0.8 % agarose gel and by spectrophotometrical analysis in the range of 220 nm to 300 nm in an UV-Vis spectro-photometer (Pharmacia).

PCR amplification

A representative PCR mixture contained 100 ng DNA; 200 µM of each dNTP; 1 µM of each primer; 50 mM KCl; 1.5 mM MgCl2; 10 mM Tris-HCl, pH 9.0; 2.5 units of Taq DNA polymerase (buffer and polymerase were purchased from Pharmacia); 1 µg T4 gene 32 protein (Boehringer Mannheim) as a stabilizer of single stranded DNA during annealing and deionized water (100µl). The mixture was overlaid with 50 µl paraffin oil. The cycle protocol consisted of one single denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 90 sec, and primer extension at 73 °C for 90 sec, a renaturation step at 72 °C for 9 min, and subsequently the samples were held at 8 °C. All experiments were conducted in a thermalcycler (Trio-Thermoblock) from Biometra which worked under block control. After the first PCR was accomplished 1 µl was used as template for the nested PCR stage. PCR pre-mixtures containing buffer, dNTP's, primers, and enzyme but no template DNA, were irradiated with UV rays at 260 nm for 10 min to avoid the amplification of artificial contaminants. The amplification products were analysed by gel electrophoresis in a 1.5 % agarose gel (Pharmacia) followed by ethidium bromide staining and UV transillumination (312 nm).

DNA amplification fingerprinting (DAF)

The PCR procedure was modified according to the demands of the hot start technique. Briefly, 25 µl reaction volume below the wax bead consisted of half the reaction buffer, primers (1 µM each), and nucleotides (200 µM each); 25 µl volume above the wax bead consisted of the second half of reaction buffer, 100 ng DNA, and 2.5 U of Taq polymerase. The cycle protocol consisted of the melting of the wax bead at 94 °C for 1 min, cooling and incubation at 20 °C for 5 min, a further denaturation step at 94 °C for 4 min followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 43 °C for 90 sec and primer extension at 73 °C for 90 sec. After a final renaturation at 72 °C for 9 min the sample was held at 8 °C.

Results

Where DNA from purified bacteria was used, the nested PCR yielded detectable amplification products down to a concentration of 1 fg DNA which corresponds to <1 bacterial genome. The results with natural soil samples are demonstrated in Fig. 1. There were no detectable amplification products after the first 30 cycles of PCR. However, after the completion of the nested PCR stage a strong signal was visible where PA gene primers were used and a somewhat weaker signal where capsule primers were used, even at a concentration of about 4 spores seeded in 100 g of soil material.

In Fig. 2 representative fragment patterns are shown generated with the DAF-primers SY265/1120. The fingerprints of all *B. anthracis* strains tested were highly conserved and clearly different from other *Bacillus* strains.

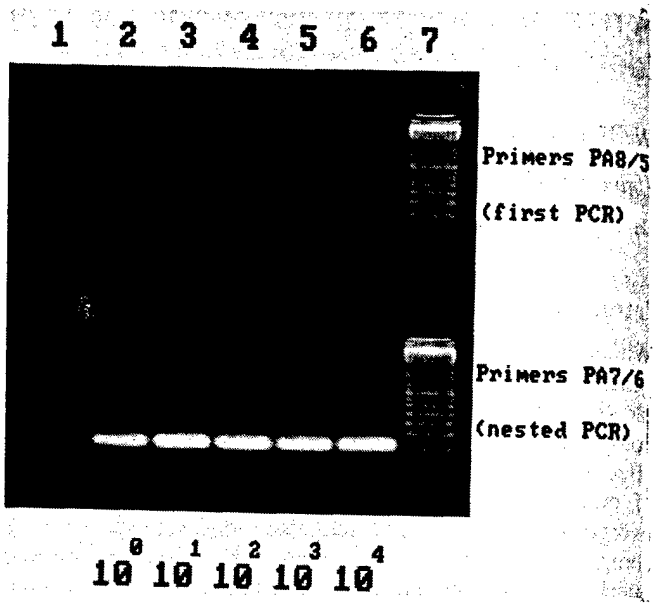

Fig. 1 Detection of the PA gene from soil samples seeded with serial log10 dilutions of *B. anthracis* spores

Lane 1 :soil sample without added *B. anthracis* ,
Lanes 2 to 6 :serial log10 dilutions of spores seeded to the soil sample,
Lane 7 :100 base pair ladder

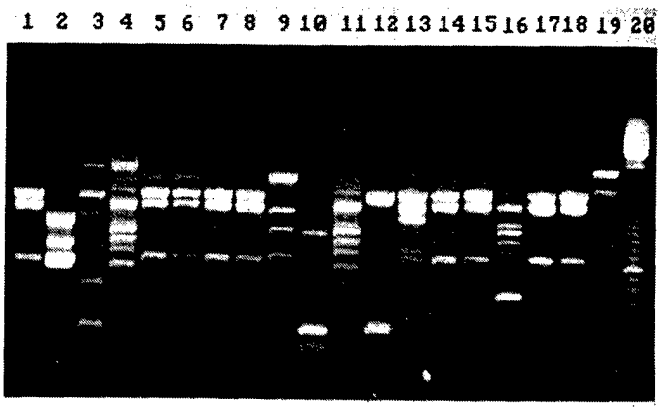

Fig. 2 DNA-Amplification-Fingerprinting (DAF) from various bacilli

Lane 1 :soil sample without added *B. anthracis* ,
Lanes 1, 5-8, 14, 15, and 17, 18 : *B. anthracis*
Lane 2 : *B. subtilis*
Lanes 3, 4, 10-13, 16, and 19 : *B. cereus*
Lane 9 : *B. megaterium*
Lane 20 : MW marker

Discussion

In this investigation a nested PCR has been developed for the detection of *B. anthracis* spores in soil samples which are heavily contaminated by inorganic compounds at the high levels which can be found at the location of a former tannery. The main problem that had to be faced was the high content of PCR inhibiting components. In fact, the enzyme activity was blocked completely if the PCR was performed with DNA preparations made from the first suspension of the soil material. Even with DNA prepared after the whole sample preparation procedure, which includes several washing steps and a 100-fold dilution of the original material, there was no detectable amplification product after 30 cycles of PCR with the outer pairs of primers. The use of the second PCR stage with an inner pair of primers substantially improves the efficiency of the PCR and makes the amplification products detectable on an agarose gel after ethidium bromide staining.

Moreover it makes the verification of the amplified products by hybridization withinner oligonucleotides or by restriction enzyme analysis unnecessary.

On the other hand, results of preliminary studies indicate that PCR products could be detected after the first PCR stage by a method called "PCR-ELISA". The labelled PCR product is hybridized to a capture probe, the hybrid is bound at the surface of a microtiter plate and may then be detected by conjugates directed against the label.

The PCR method enables investigators to benefit from the use of a non-selective enrichment step. This is in contrast to the conventional culture method, where the huge number of competing bacteria, grown in a nonselective broth, make a detection of *B. anthracis* on the conventionally used selective agar plates nearly impossible.

The method described allows the detection of less than 10 spores per 100 g of the original soil sample. This is about 10^7-fold more sensitive than the conventional culture diagnosis. With this method we were able to demonstrate in some cases the contamination of soil from a former tannery site in Germany with *B. anthracis*. A "positive control sample", consisting of 100 g representative soil material seeded with about 4 bacterial spores, was processed under identical conditions with each of the several hundred samples which were investigated until today. In all cases this sample yielded a positive result.

Because of the possibility that a plasmidless *B. anthracis* could arise under laboratory culture conditions we searched for a method to confirm such strains as really being *B. anthracis* . For this reason oligonucleotides from the secY-gene of *B. subtilis* were generated as PCR primers. The gene belongs to a gene family responsible for the protein excretion mechanism in *B. subtilis*. Under PCR-conditions where the annealing of these primers was allowed to only weak homologeous target sequences, we could generate fragment patterns which were highly conserved within *B. anthracis* strains and clearly different from other *Bacillus* strains. The amplification fingerprints yielded by these primers were not influenced, whether the plasmids were both present or not.

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Detection of the vegetative form of *Bacillus anthracis* in soil by PCR

A. SJÖSTEDT[1], U. ERIKSSON[1], V. RAMISSE[2] and H. GARRIGUE[2].

[1]Department of Microbiology, National Defence Research Establishment, S-901 82 Umeå, Sweden and [2]Laboratoire de Biochemie Immunochemie, Centre d'Études du Bouchet, 91710 Vert-Le-Petit, France.

Summary and introduction

Bacillus anthracis, the etiological agent of the highly infectious disease anthrax, has two life forms, as a vegetative cell and as a spore. It is believed that the ability of the bacterium to form spores is critical for maintaining its infectious cycle. However, it has been suggested that *B. anthracis* can replicate in some soils, resulting in a high risk for animals and humans to acquire the infection in these areas[1]. We hear investigated the possibility to rapidly detect the vegetative form in soil by use of the polymerase chain reaction (PCR).

Materials and methods

B. anthracis 7703, the Sterne strain (pX01$^+$, pX01$^-$), was obtained from Institut Pasteur. *B. anthracis* 4229 (pX01$^-$, pX01$^+$), the Pasteur vaccine, was obtained from ATCC.

Soil samples were prepared as previously described[2]. Briefly, samples were ground in a mortar and mixed with silica powder (1:9) and transferred to Ultrafree-MC filters (low-binding Durapore, 0.22 µm, Millipore Inc.) and subjected to 3 freeze-thaw cycles. Then, 500 µl of 10 mM PBS containing 1% (w/v) polyvinylpolypyrrolidone (Sigma Chemical Co. Mo), 228 µl TE-buffer, 50 µl 10% SDS, and 5 µl proteinase K were added, followed by an incubation at 40 °C for 10 min. Next, 110 µl 5M NaCl and 88 µl 10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl were added, followed by an incubation at 65 °C for 10 min. One ml of phenol was added and samples were eluted using first 1M NH_4Cl and then 3M NH_4Cl. 100 µl of the eluates were further concentrated on a microspin column (SR-400 HR, Pharmacia Biotech). Ten µl of the final eluates was used directly in the PCR, performed as previously described[2].

Results and discussion

Primers shown in Table 1 were based on previously published sequences available in the EMBL data base of the genes encoding *cap* and *lef*. The specificity and sensitivity of PCR-based amplification using these primers were determined. The detection limit was 6 x 10^0 bacteria/assay for primer pair BA17-BA20 (specific to the *cap* operon) and 2 x 10^1 for primer pair BA54-BA6 (specific to the *lef* gene). Next, we determined the specificity of these primer pairs by investigating if they enabled amplification of DNA originating from other species of *Bacillus* or other genera. It was found that no amplification of DNA of 8 *Bacillus* strains or 6 other strains representing various Gram-positive and Gram-negative genera resulted using either primer pair BA54-BA6 or BA17-BA20. Thus, the two primer pairs used appeared to be suitable for subsequent experiments as they showed no cross-reactivity with genetic material from species other than *B. anthracis* and allowed a reasonably sensitive detection.

It is well documented that before the PCR can be applied, many types of samples require extensive treatment to remove inhibitors. This is particularly true for soil samples containing humic substances[3]. Thus, we first investigated the usefulness of various methods for extracting total DNA from the soil samples and then amplifying *B. anthracis*-specific DNA from the resulting material. First 5 horizons were sliced from a sample of sandy iron podzol. We found that after addition of *B. anthracis* cells, DNA could efficiently be extracted according to a previously published methods[2] and amplified by PCR. This approach allowed a detection limit of 3 x 10^1 bacteria/mg silica. For the soil horizons, the detection limit varied between 3 x 10^1 and 3 x 10^3 /mg soil. Next, various other types of soil were investigated. At random locations, samples were collected from swamp. lawn, moss, meadow, cultivated soil, and litter. In these experiments, it was found that effective amplification was only achieved in 2 out 8 soil types.

Conclusion

We have developed a PCR-based method that allows sensitive detection of *B. anthracis* in certain soil types. However, extended analysis showed that the method was not generally applicable, in as much as no amplification was achieved in other types of soil. We are at present investigating the possibility that a modified method will have a wider application.

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Table 1. Summary of primers specific to genes of *B. anthracis* used in the present study.

Designation	Specificity and location	Sequence	T_a
BA54	*lef*, 606-585	5'-CAT ACC TAC ATC ACC ATG ACC G	66
BA6	*lef*, 1941-1921	5'-TAA ATC CGC ACC TAG GGT TGC G	64
BA17	*cap*, 1230-1249	5'-GAA ATA GTT ATT GCG ATT GG	54
BA20	*cap*, 2102-2083	5'-GGT GCT ACT GCT TCT GTA CG	62

Multiplex PCR assay for identification of *Bacillus anthracis* and differentiation from *Bacillus cereus* group bacteria

V. RAMISSE[1], G. PATRA[1,2], V. VAUDE-LAUTHIER[1], P . SYLVESTRE[1], J. THERASSE[1] and J.-L. GUESDON[2].

[1] *Laboratoire des Biotechnologies, Centre d'Etudes du Bouchet, B.P. no 3, 91710 Vert-Le-Petit, France*
[2] *Laboratoire de Prédéveloppement des Sondes, Institut Pasteur, 75724 Paris Cedex 15, France.*

The purpose of this work was to develop a single step PCR assay for identification of potentially virulent strains of *Bacillus anthracis*. Thus, genes encoding recognised virulence factors (*lef, cya, pag*, and *cap* localised on plasmids pXO1 and pXO2 respectively) [1, 2, 3, 4] were chosen as specific sequences representative of virulent *B. anthracis*. In addition the sequence Ba813, a *B. anthracis* specific chromosomal marker isolated from a genomic library[5], was used to confirm species.

Two primer pairs were selected from *lef, cya, pag,* and *cap* sequences by computer analysis and were synthesized by Genset (Paris, France) or using the Cyclone DNA synthesizer (Millipore Waters). Primer sets were pooled so that all of the four targeted genes were amplified once (16 combinations tested consisting of 8 sets). PCR was performed with GeneAmpTM PCR system 9600 and under the following conditions: total volume (25 µl), total genomic DNA from a fully virulent *B. anthracis* strain (~100 ng), oligonucleotides (0.5 µM), dNTPs (200 µM), $MgCl_2$ (1.5 mM), Taq-DNA polymerase Boehringer (1 U), cycling parameters (5 min at 94°C for denaturation, followed by 30 cycles: 30 sec at 94°C, 30 sec at 58°C, 30 sec at 72°C). PCR products were detected by ethidium bromide staining after agarose gel electrophoresis. Four fragments with the expected size were successfully amplified using nearly all combinations. On the basis of band intensity and electrophoretic resolution, the combination employing 67/68, 25/26, 3/4 and 57/58 primer pairs (Table 1) was shown to be the most efficient. Thus, this combination was used for further experiments including R1/R2 primers which are specific for Ba813 sequence (Table 1). Multiplex PCR assays targeting both plasmid and chromosomal markers were successfully carried out by using 67/68, 25/26, 3/4, 57/58 and R1/R2 oligonucleotide primers.

The specificity of this multiplex assay using five primer pairs was tested against 31 *Bacillus anthracis* strains obtained from several French culture collections and 11 *Bacillus* strains encompassing 10 other species (Figure 1). The results obtained demonstrate the value of the oligonucleotides designed from plasmid and chromosomal markers of *B. anthracis* and their applicability for identification of avirulent and potentially virulent strains of *B. anthracis*. Our multiplex PCR assay can easily distinguish $pXO1^-/2^-$ strains from *B. cereus*. This PCR will be useful for a more in-depth survey to identify unambiguously isolates resembling *B. anthracis*[6,7], and could be used when a simple and rapid identification system is needed. In conclusion, this multiplex PCR assay could contribute to surveillance procedures in the industrial setting as a rapid method for diagnosing anthrax.

Table 1. Specific oligonucleotide primers used in this work.
(*)Positions are given according to published sequences.

Loci	Primer sets	Positions*	Products (bp)	References
pag	67/68	1925-1944/2652-2671	747	1
cya	25/26	1459-1478/ 1990-2004	546	2
lef	3/4	1238-1258/1599-1622	385	3
cap (C)	57/58	1603-1622/1847-1868	264	4
Ba813	R1/R2	227-249/98-120	152	5

* to identify unambiguously isolates resembling *B. anthracis*.

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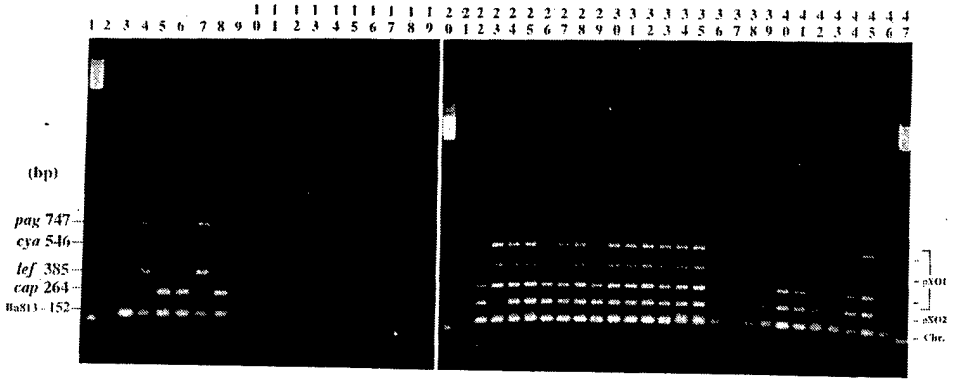

Figure 1. Results of the amplification of total genomic DNA (~100ng) by 67/68, 25/26, 57/58, 3/4 and R1/R2 primer pairs. 123bp ladder (1,20,47)), negative control (2, 21), *B. anthracis* Sterne 7700 (3), Sterne 7702 (4), ATCC 4229 (5), ATCC6602 (6), Cepanzo (7), Davis TE702 (8), *B. subtilis* ATCC6051 (9), *B. pumilus* ATCC7061 (10), *B. licheniformis* ATCC14580 (11), *B. stearothermophilus* ATCC7953 (12), *B. thuringiensis* ATCC10792 (13), *B. sphaericus* ATCC14577 (14), *B. alvei* ATCC6344 (15), *B. megaterium* ATCC14581 (16), *B. pasteurii* ATCC11859 (17), *B. cereus* ATCC14579 (18), *B. subtilis* ATCC9372 (19), *B. anthracis* 227 (22), 957 (23), 170 (24), 300 (25), 779 (26), 832 (27), 663 (28), 376 (29), 846 (30), 256 (31), 582 (32), 282 (33), 85 (34), 576 (35), 955 (36), 554 (37), 657 (38), 516 (39), 346 (40), 552 (41), 470 (42), 575 (43), 204 (44), 893 (45) and 69 (46).

Fingerprinting of *Bacillus anthracis* by pyrolysis mass spectrometry

P.R. SISSON and N.F. LIGHTFOOT

Newcastle Regional Public Health Laboratory, General Hospital, Westgate Road, Newcastle upon Tyne NE4 6BE, UK

Summary

Pyrolysis mass spectrometry (PyMS) differentiated 13 strains of *Bacillus anthracis* from 13 isolates of *B. cereus* and *B. thuringiensis*. Moreover, PyMS-analysis differentiated multiple subcultures of a single strain of *B. anthracis* from 12 other strains of the same species.

PyMS is a simple, rapid and relatively inexpensive technique which may be of great value in distinguishing anthrax isolates from closely-related microorganisms. Fingerprinting of *B. anthracis* strains by PyMS could be a useful tool in epidemiological studies of outbreaks of anthrax and merits further study.

Introduction

Pyrolysis mass spectrometry (PyMS) has been shown to give reliable strain differentiation of bacteria causing outbreaks of human disease[1-3] and has been particularly useful for those genera for which typing systems are relatively undeveloped[4,5]. It has been shown to give rapid, reliable and accurate inter-strain comparisons of Bacillus spp and Clostridium spp implicated in food-borne infections[6].

Preliminary studies demonstrated the ability of PyMS to differentiate strains of *B. anthracis* from *B. cereus*[7]. An assessment of the discriminatory power of PyMS was complicated by the protocols necessary for handling containment level 3 organisms and by the relative paucity of conclusive epidemiological data. In this study, the techniques used for preparing the organisms for analysis by PyMS have been refined, and the ability of PyMS to detect an outbreak cluster of *B. anthracis* isolates amongst a collection of closely related strains has been investigated.

Methods and materials

Bacterial strains

The thirteen strains of *B. anthracis*, ten strains of *B. cereus*, two strains of *B. thuringiensis* and one of *B. thuringiensis israelensis* used in this study (Table) were all supplied by the Anthrax Section, CAMR. Of the *B. anthracis* strains, six had been isolated from samples taken in Etosha National Park in 1977, and a further five had been isolated from the same area in 1991. One of the remaining two strains was an environmental isolate from a decommissioned laboratory and the other was isolated from bonemeal in the UK. In order to ensure the ability of PyMS to detect a cluster of isolates of a single strain of *B. anthracis*, one strain (ASC 72) was subcultured 5 times on initial receipt, and thereafter each subculture was treated as if it were a separate isolate.

Preparation of samples for PyMS

Duplicate subcultures of the isolates were made in 10 ml of nutrient broth (Oxoid) and incubated at 37°C for 6 hours. The broth cultures were then autoclaved at 121°C for 20 minutes. The following day, the sterilised broth cultures were centrifuged at 3,000 x g for 15 min. The supernatants were discarded and approximately 5 µl of each deposit were used to load each of three Ni-Fe pyrolysis foils (Horizon Instruments Ltd. Heathfield, Sussex,UK) suspended in pyrolysis tubes (Horizon Instruments). The foils were dried at 80°C for 10 min prior to pyrolysis at 530°C for 4 seconds on a Horizon Instruments 200X pyrolysis mass spectrometer, as previously described[8,9]. The integrated ion counts in the mass range m/z 51-200 were collected on a floppy disk, the results of the replicates of each subculture being labelled as a separate group.

Data analysis

After normalization to correct for variations in sample size, the spectral data were used in principal component (PC) and canonical variate (CV) analysis[10]. The analysis data were represented on an ordination diagram of PCCV1 versus PCCV2. The table of Mahalanobis distances was used in an unweighted pair group method with averages (UPGMA) analysis to produce similarity dendrograms[11]. In addition, the spectral data from the two subcultures of each isolate were summated and reanalysed to create PyMS groups containing data from six replicate foils. These groups were labelled by PyMS with lower case letters.

The relationships between pyrolysis groups, and hence between isolates, can be inferred from the relative proximity of their data points on the ordination diagrams or similarity dendrograms[12]. Data for isolates which were distinguished as outliers on ordination diagrams or similarity dendrograms were edited from the dataset. This was then reanalysed to examine in greater detail the relationship between more closely related isolates[12].

Data from isolates clustered by the standard PCCV analysis were used in a series of analyses in which the principal component canonical variate mean (PCCV mean) of the data from each isolate was compared directly with the PCCV mean of the data from each other isolate. The differences between the PCCV means for each comparison were used to construct a similarity matrix ordered by UPGMA analysis, as previously described[13]. Provided that the results obtained in the comparisons of duplicate subcultures conform to a normal distribution, the values approximate to those of χ^2 distribution with one degree of freedom, and values of 3.84, 6.63 and 10.84 are taken to indicate 95%, 99% and 99.9% confidence limits respectively[13].

Results

The results of the first analysis of the dataset, in which the spectral data from duplicate subcultures have been summated and meaned, are shown in Figure 1. The similarity dendrogram is comprised of two main clusters. The top clusters included eight of the ten strains of *B. cereus* (r, w, v, x, t, u, y, z), together with the two strains of *B. thuringiensis* (3, 5) the strain of *B. thuringiensis israelensis* (7) and two strains of *B. anthracis*; the multiple subcultures of ASC 72 (a-e) and ASC 197 (o).

The lower half of the dendrogram entrained 11 of the 13 strains of *B. anthracis* (f, p, q, g, I, n, j, k, l, m, h) and two strains of *B. cereus* (s, 1).

When each cluster from this dendrogram was analysed separately, the two strains of *B. cereus* (s, 1) were clearly differentiated from the 11 *B. anthracis* isolates which were shown to be distinguishable by PyMS (data not shown).

The ordination diagram of the analysis of the top cluster from the first dendrogram (Figure 2) showed a clear distinction between the isolates of *B. cereus* and those of *B. anthracis* (a-e, o). The PyMS spectral data for the *B. cereus* strains were edited from the dataset, and it was reanalysed using data pertaining to only the *B. anthracis* isolates ASC 72 and ASC 197. PyMS analysis clearly differentiated isolate ASC 197 (o) from the fake clone created by using multiple subcultures of ASC 72 (a-e) (Figure 3). This distinction is clearly demonstrated by the similarity dendrogram (Figure 4) derived from analysis of the separate subcultures of each isolate (ie using only triplicate data for each PyMS group), and in the similarity matrix (Figure 5).

In order to investigate further the ability of PyMS to discern an outbreak cluster of multiple isolates of a single strain of *B. anthracis*, a further analysis using spectral data from only *B. anthracis* isolates was performed. The resulting similarity dendrogram (Figure 6) again shows the fake clone of ASC 72 (a-e) clustered together and distinguished from the remaining isolates.

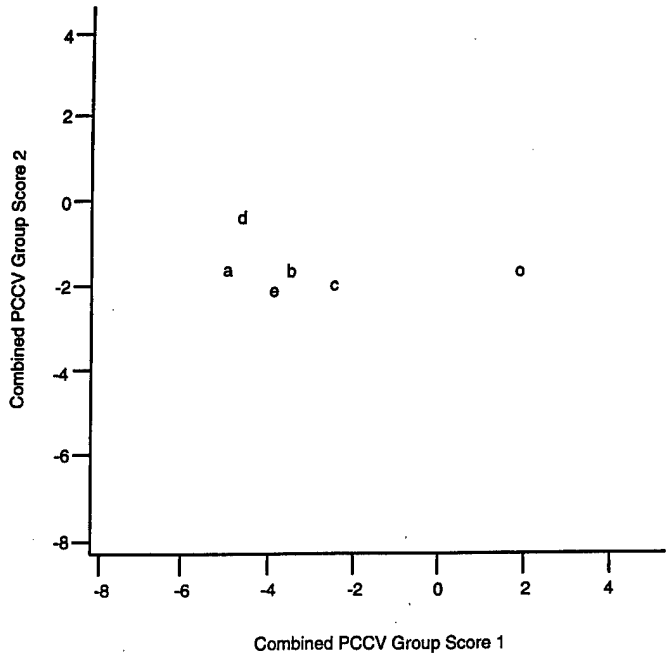

Figure 3. Ordination diagram of spectral data from PyMS analysis of 5 replicate subcultures *B. anthracis* strain ASC 72 (a-e) and one of *B. anthracis* strain ASC 197 (o).

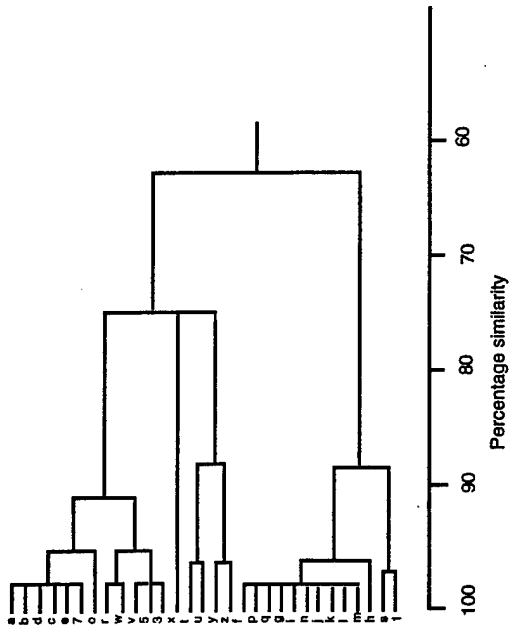

Figure 1. Similarity dendrogram derived from PyMS analysis showing percentage similarity of 26 isolates of *Bacillus* spp as designated in Table 1.

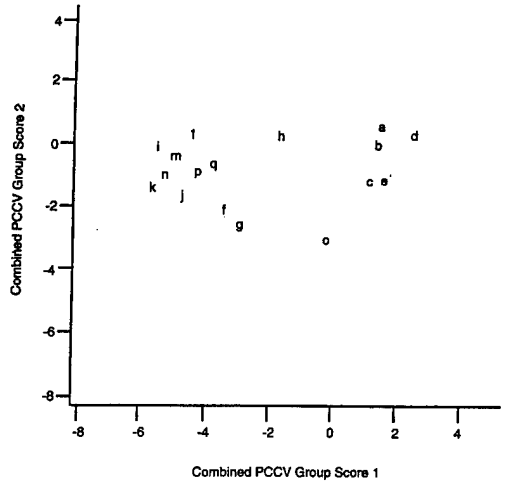

Figure 2. Ordination diagram of spectral data from PyMS analysis of 13 *B. anthracis* isolates, as designated in Table 1, including one sampled from 5 subcultures to create a clone (a-e).

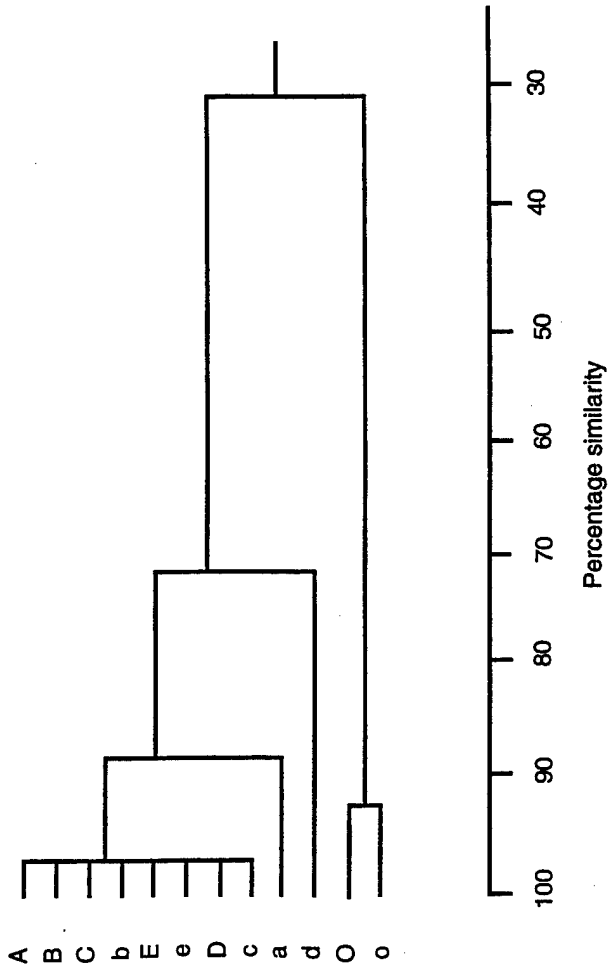

Figure 4. Similarity dendrogram derived from PyMS analysis showing percentage similarity of duplicate subcultures of 5 replicate cultures of *B. anthracis* ASC 72 (Aa-Ee) and duplicate subcultures of *B. anthracis* strain ASC 197 (o).

a	X					
b	3.11	X				
d	1.22	1.98	X			
e	1.66	3.75	2.39	X		
c	2.37	3.79	1.86	1.87	X	
o	17.25	14.17	13.62	22.66	12.72	X
	a	b	d	e	c	o

Figure 5. Similarity matrix compiled from the results of individual comparisons of PCCV means of pyrolysis mass spectra of five replicate cultures of *B. anthracis* strain ASC 72 (a-e) and one culture of *B. anthracis* strain ASC 197 (o).

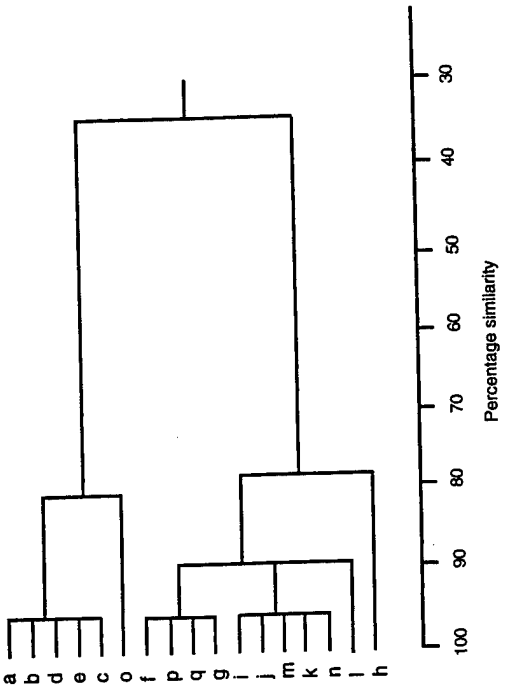

Figure 6. Similarity dendrogram derived from PyMS analysis showing percentage similarity of 13 strains of *B. anthracis*, as designated in Table 1.

Discussion

B. anthracis remains a potential biological weapon of aggression and considerable defence-related research is directed towards it[14]. It is still difficult to distinguish isolates of *B. anthracis* rapidly and reliably from others of closely-related species.

Early studies with PyMS demonstrated it to be an accurate method for characterising many bacteria including those belonging to Bacillus spp[1-6]. Poor discrimination of anthrax strains by PyMS led other workers to suggest that the technique might be unable to differentiate this species[15]. It has since been shown that PyMS could distinguish *B. anthracis* strains from isolates of *B. cereus*, despite autoclaving the cultures at high temperatures prior to pyrolysis in order to render them safe thereafter to be processed in a containment level 2 laboratory[7]. As a phenotypic method, PyMS characterisation is profoundly affected by the conditions used to prepare the microorganisms for PyMS analysis[16,17].

In this report, 13 strains of *B. anthracis* were differentiated by PyMS from 10 strains of *B. cereus* and 3 of *B. thuringiensis*, but sequential analysis of the PyMS spectral data was required to achieve full separation. The lower autoclaving temperature used has improved the discrimination. Eleven of the 13 *B. anthracis* isolates clustered together on the first analysis. It remains likely, however, that pre-exposure of the microorganisms to temperatures in excess of 100°C is causing some thermal degradation, leading to a blunting of the level of PyMS-discrimination. Alternative methods of killing the anthrax isolates, such as exposure to cobalt-irradiation, should be less destructive of their cellular architecture, preserving the discriminatory power of PyMS in line with that achieved with other bacterial species pyrolysed directly from culture media.

PyMS did detect multiple isolates of a single strain of *B. anthracis* from other anthrax strains. Further work with field isolates, having good epidemiological evidence of relatedness, which have been characterised by other methods, is needed to substantiate this result. PyMS has great promise in studying the natural history of anthrax. It is a simple, rapid and inexpensive technique which is equally effective at characterising a wide range of other pathogens of military significance.

Further studies will include the application of artificial neural network (ANN) analysis to PyMS spectral data. Previous studies have shown that PyMS-ANN analysis can differentiate *Mycobacterium tuberculosis* isolates from those of *M. bovis*[18], and can be used to distinguish Verocytotoxin-producing strains of *Escherichia coli* from non-toxigenic strains of the same serogroups[19]. Preliminary work using the PyMS data presented above, suggests that ANNs could be developed to distinguish *B. anthracis* strains from those of *B. cereus* and similar species in a single analytical process. This work is being actively pursued.

In conclusion, the current report has confirmed the PyMS can be used to fingerprint *B. anthracis* isolates. Improvements to the necessary protocols for handling a containment level 3 organism could result in improved PyMS discrimination and merits further study.

Table: 1 Details of the 26 strains of *Bacillus* spp examined by pyrolysis mass spectrometry

***Bacillus* isolate**		**PyMS Designation**
B. anthracis	ASC 72 (1-5)	a, b, c, d, e
	ASC 73-77	f, g, h, i, j
	ASC 179	k
	ASC 194-198	l, m, n, o, p
	ASC 205	q
B. cereus	10 strains	r, s, t, u, v, w, x, y, z, 1
B. thuringiensis	2 strains	3, 5
B. thuringiensis israilensis	1 strain	7

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Fingerprinting *Bacillus anthracis* strains

I. HENDERSON

Centre for Applied Microbiology and Research, Salisbury, Wiltshire, SP4 0JG, United Kingdom

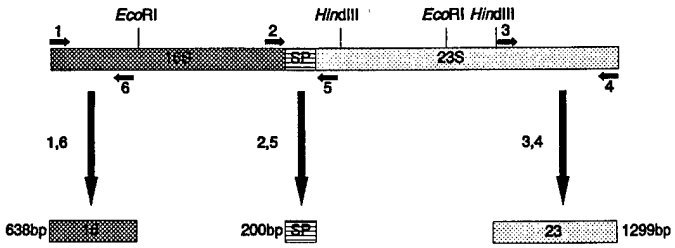
Summary

Polymerase chain reaction (PCR) based fingerprinting studies of *Bacillus anthracis* have identified few regions of chromosomal heterogeneity between strains. Similarly, there is little evidence to suggest that *rrn* genes would possess any useful intrinsic sequence variation for discriminatory purposes. In this study, *rrn* sequences have been used to look for restriction fragment length polymorphisms (RFLP s) in rRNA operon flanking sequences. RFLP s detected 3 to the 23S gene using *Hin*dIII digested chromosomal DNA have proven the most reliable means of detecting variation between strains and have made it possible to the species into six groups. Strains probed with 16S gene sequences have shown the greatest variation between strains, particularly in response to routine laboratory manipulation, such as animal passage and vaccine production. Fingerprint patterns are highly conserved in the species compared to a small number of *B. cereus* group strains (*B. cereus, B. mycoides and B. thuringiensis*) analysed with the same probes. This allows the specific identification of *B. anthracis* from these other related species. Surprisingly, a probe based on the 16S-23S intergenic region of *B. anthracis* rrn operons was capable of sub-differentiating a small proportion of *B. anthracis* strains but not other members of the *B. cereus* group.

Introduction

Epidemiological and strategic tracing exercises with *B. anthracis* are dogged by an inability to suitably discriminate between individual strains. This is due to the phenotypic coherency within the species and its similarity with other closely related members of the *B. cereus* group (*B. cereus, B. mycoides and B. thuringiensis*), which make conventional biochemical, immunological and phage differentiation systems inapplicable.

Molecular genetic fingerprinting applications have shed some light on the problems posed by *B. anthracis*. PCR fingerprinting techniques have not detected any gross chromosomal structural differences between a diverse set of *B. anthracis* strains[7,8], supporting DNA homology data for the species[3]. In contrast, other members of the group show significantly greater heterogeneity at the chromosomal level, even between strains.



Primer 1: 5 -GAGAGTTTGATCCTGGCTCAG-3
Primer 2: 5 -GCGGCTGGATCACCTCCTTTC-3
Primer 3: 5 -AGGCAAATCCGCTTATCG-3
Primer 4: 5 -ATGGTTAAGTCCTCGATC-3
Primer 5: 5 -CAAGGCATCCACCGTGCGCCC-3
Primer 6: 5 -GCACTCAAGTATCCCAGT-3

Figure 1: Putative structure of the rrn operon of *B. anthracis*, and the locations of oligonucleotide primers used to synthesise the fingerprinting probes, fragments 16, 23 and SP. Sequences were taken from those published for *B. anthracis* rRNA genes 1,2.

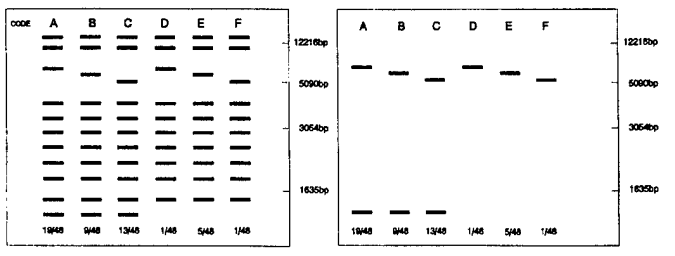


Figure 2: Computer graphics summarising fingerprinting data for *Hin*dIII/fragment 23. Panel 1 shows all bands produced with the probe; for clarity, panel 2 shows just the variable regions of fingerprints. The fingerprint codes with this probe are A - F and form the last letter of the code in Table 1.

Despite a lack of DNA sequence data for *B. anthracis* , there are still several likely targets for fingerprinting studies. Differences observed between strains at the level of virulence may relate directly to subtle differences in toxin genes and their regulatory elements. To date all genes that influence virulence are located to plasmids, which may be unstable in the environment[21]. Chromosomal sequences would therefore be more suitable for epidemiological markers. Conventional targets for DNA fingerprinting are the rrn genes because of their pivotal role in the control of gene expression. DNA sequence analysis of these genes in the *B. cereus* group have shown that very few differences exist between members, suggesting that the genes would be very unlikely to display sequence heterogeneity between *B. anthracis* strains[1,2]. However, these genes, because of their homogeneity, provide an excellent platform for assessing sequence variation in their flanking regions.

This study shows that *B. anthracis* strains can be fingerprinted and sub-differentiated reliably by probes directed at the 23S rRNA gene that detect restriction fragment length polymorphisms (RFLPs) 3 to it. This study has also indicated that RFLP s 5 to the 16S gene are susceptible to variation after routine laboratory handling of strains such as animal passage of vaccine production. It was also possible to detect variation between *B. anthracis* strains using the 16S-23S intergenic spacer DNA as a probe that conversely failed to detect variation in the other species of the *B. cereus* group.

Materials and methods

Bacterial strains

Strains of the *B. cereus* group used in this study are presented in Table 1. *B. anthracis* was isolated from environmental samples (soil, tissue) as previously described[20]. Strains were also analysed for protective antigen (PA) and plasmid content[21].

DNA isolation, digestion and blotting

Total cellular DNA was isolated from all *B. cereus* group bacteria as previously descibed[7]. Digestion of chromosomal DNA with the enzymes *Eco*RI and *Hin*dIII was carried out according to the manufacturers instructions (NBL, Cramlington, UK). DNA digests were analysed in 0.7 % agarose gels at 2.5 V/cm under standard conditions[14]. DNA fragments were transferred on to charged nylon membranes (Hybond H^+; Amersham International, UK) according to manufacturers instructions, and stored at 4°C prior to use.

Isolation of DNA probes

DNA sequences from 16S, 23S and the 16S-23S intergenic spacer were isolated using PCR (Fig. 1) from *B. anthracis* ASC 1 (NCTC 8234). Oligonucleotides, taken from the published sequences for the *rrn* genes of *B. anthracis* [1,2] were synthesised using an Applied Biosystems 380B DNA synthesiser by the automated phosphoramidite coupling method. PCR reaction mixtures (100 µl) contained 150 ng of template DNA, 1.25 mM each of dATP, dCTP, dGTP and dTTP, 3.0 mM Mg^{2+}, and 2 µM of each nucleotide primer. Template DNA was initially denatured at 96°C for 5 min. followed by 30 cycles of (i) 95°C for 2 min., (ii) 53°C for 2 min. and (iii) 72°C for 2 min., in an MJ Research PTC 100 thermocycler. Mineral oil overlays, used to minimise evaporation in the reaction tubes were removed after amplification by freezing the samples to -20°C, and removing the oil by aspiration. Samples were then analysed by electrophoresis in low melting point agarose gels[14].

DNA probe labelling and hybridisation

PCR products in low melting point agarose were placed in 1.5 ml sterile eppendorf tubes with sterile distilled water to a volume equivalent to 3 ml/g of agarose. The agarose was melted at 70°C and the mixture dispensed into aliquots and stored at -20°C prior to use. Each aliquot was used in the synthesis of [α-^{32}P]dATP labelled DNA probes using a Megaprime DNA labelling kit (Amersham International, UK) and pentanucleotides (Pharmacia LKB, UK) as primers. Hybridisation (overnight) was performed in 10 ml of 7 % (w/v) SDS, 1 % (w/v) BSA and 260 mM sodium phosphate buffer (pH 7.2)[22], after prehybridisation of the blot in the same solution for a minimum of 30 min. Blots were washed 3 x 15 min. (in 2x SSC, 1 % (w/v) SDS) at 58°C, and then autoradiographed against Kodak XAR-5 film for up to 4 h at -70°C.

Cloning and sequencing of 16S-23S intergenic spacers

PCR products amplified from primers 2 and 5 (Fig. 1) were cloned using a commercially available kit (TA™ Cloning Kit, Invitrogen) using *Escherichia coli* INVαF (F', *endA1, recA1, hsdR17*(r_k^-,m_k^+),λ^-, *supE44, thi-1, gyrA96, relA1, ϕ80Δlac ΔM15, Δ(lacZYA-argF)U169, deoR)* as host. Recombinant plasmids were isolated using Wizard™ minicolumns (Promega, UK) and sequenced by the dideoxynucleotide chain termination method using modified T7 DNA polymerase (Pharmacia LKB, UK)[18]. DNA sequences presented from each strain are derived from three individual PCR/cloning steps to ensure fidelity.

Nucleotide sequences

The nucleotide sequences of *B. cereus* group 16S-23S intergenic spacers have been submitted to the EMBL/Genbank database and have the accession numbers L42532-L42536.

Results and discussion

Fingerprinting results using the 16S, 23S and 16S-23S intergenic probes (Fig.1) against *B. anthracis* total DNA digested with *Hin*dIII and *Eco*RI are summarised in Table 1. Each strain has a five letter code according to the fingerprints obtained with each enzyme/probe combination; this is fully explained in the footnote to the table. Although three probes and two enzymes were used in the study, each strain only has a five letter code. This is because the fingerprint patterns for *Hin*dIII/fragment 16 and *Hin*dIII/fragment SP are identical as would be predicted from the putative structure of the rrn operon of *B. anthracis* (Fig. 1).

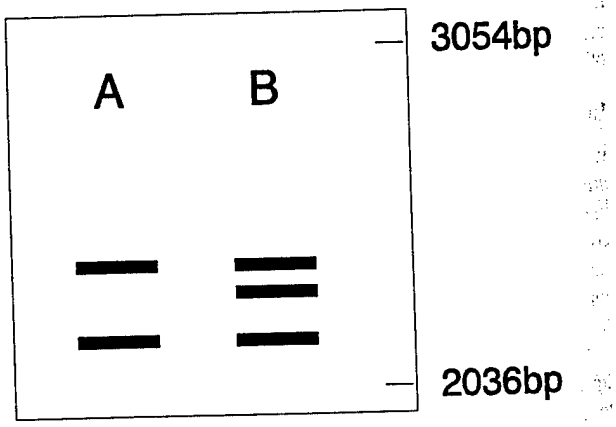


Figure 3: Computer graphic summarising fingerprinting data for *Eco*RI/fragment SP. Lane 1 includes all strains listed in Table 1 except for Lane 2, ASC 79, ASC 91 and ASC 93.

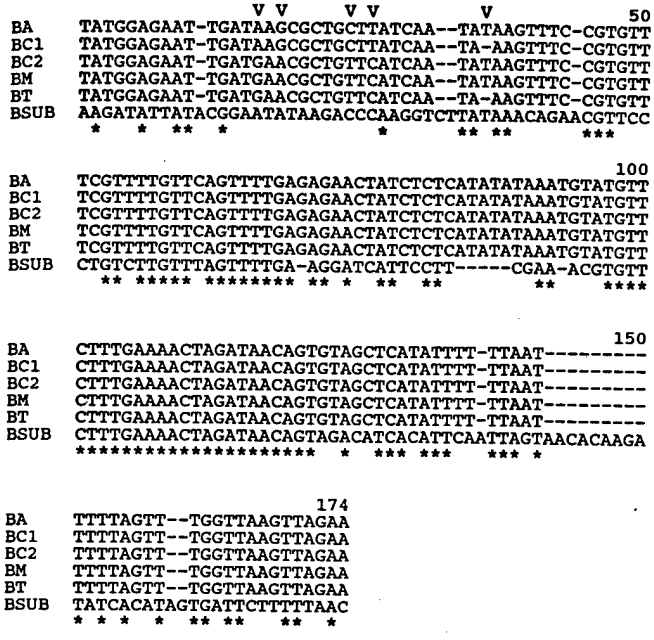


Figure 4: Nucleotide sequence alignments[9] of 16S-23S intergenic spacers from the *B. cereus* group compared with *B. subtilis*. Asterisks indicate sequence identity. Numbers in brackets are the EMBL/Genbank accession numbers. BA: *B. anthracis* (L42536); BC1: *B. cereus* (L42532); BC2: *B. cereus* (L42533); BM: *B. mycoides* (L42535); BT: *B. thuringiensis* (L42534); BSUB: *B. subtilis*.

The enzyme *Hin*dIII was the most discriminatory, and in combination with fragment 23 allowed the *B. anthracis* strains to be sub-differentiated into six groups, labelled ENV, VOL, VAX, BRZ, ZIM and PAS (Fig. 2). VOL and VAX group members are the most widely used strains used at CAMR for vaccine production (VAX) and research (VOL). The consistency of the fragment 23 probe results for these strains is therefore surprising. This probably results from the fact that sequences 3 to the 23S gene is dominated by 5S rRNA and tRNA genes which because of their role in gene expression are likely to be conserved. This is in contrast to the results for the fragment 16 probe for some of the isolates in the two groups which show variation after laboratory handling (see Table 1). Sequences in this region may be expected to be under lower selective pressure than those 3 to the 23S gene unless they are found close to or at the host chromosome origin of replication.

It is interesting to note that the 16S and 23S probes have shown that the type strain for the species ASC 6 (NCTC 10340) is somewhat different to the other strains in the study. The original isolation of the Vollum type strain was not done in this laboratory, and its handling prior to deposition in our culture collection is unknown. However, subculture of the organism (see ASC 2, 3, 184, 185 and 328 - see Table 1) appears to select for the 16S based RFLPs observed in the ENV group (i.e. code A). These RFLPs would therefore be unsuitable for tracing exercises. A similar pattern is observed with Ames and its re-isolates in the VAX group. Other RFLP patterns observed in the BRZ, ZIM and PAS groups have not been tested for their stability in the laboratory, but their rarity compared to the rest of the strains in the study suggests that these are more permanent changes. Fragment SP probing of *Eco*RI digests gave unexpected results. If the intergenic spacer was unique, then a single band on probing with SP would be anticipated. However, almost all *B. cereus* group strains gave a double band pattern (Fig. 3), suggesting the presence of alternative spacers in the group. The exceptions to this are three *B. anthracis* strains that gave three bands. These alternative banding patterns are likely to represent intergenic spacers that have DNA sequence homology with the spacer probe but which, as in other micro-organisms, may also have sequences for tRNA genes inserted in them.

To rule out sequence heterogeneity in the SP probe itself, spacers were isolated by PCR from representative strains of the *B. cereus* group using reaction conditions identical to those that were used to isolate fragment SP. These were then cloned and sequenced (Fig. 4). Only a single band was produced with the PCR conditions used, and no other type of insert was detected in *E. coli* transformants. All spacers isolated from *B. anthracis* strains had identical sequences. Two different spacer sequences were identified for *B. cereus*, and a single sequence each was detected for *B. mycoides* and *B. thuringiensis* respectively. These results indicate that there are at least two spacer types in the *B. cereus* group with sequences similar to the SP probe, and, in the case of the ENV 1 *B. anthracis* subgroup, three types. The SP probe would detect all spacers sequenced for the group, but this probably only represents one of the bands on the *Eco*RI/fragment SP fingerprints. As with other species such as *B. subtils*, spacer sequences have been found to contain tRNA gene sequences. The presense of such sequences in the spacers detected with fragment SP would account for the small differences in molecular weight observed for the different bands of *Eco*RI/fragment SP fingerprints.

In conclusion, as found with the PCR fingerprinting methods[7,8], *B. anthracis* strains appear to be highly conserved at both phenotypic and genotypic levels. Results suggest a clonal population that is only just beginning to show the signs of independent variation. This may reflect that as an obligate pathogen, *B. anthracis* may only multiply when the opportunity to infect a suitable host arises and that for any one strain this may be a rare event. Alternatively, it may also indicate that the evolution of the organism from a *B. cereus*- like precursor has occurred only relatively recently.

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Table 1: Origins and characteristics of *B. cereus* group strains used in this study.

ASC	Fingerprint code[b]	Fingerprint group	Other Designation[c]	Origin and year of isolationd	Ref
15	AAAAA	ENV	ATCC 938	Human, UK, 1931	5
27, 28, 29, 30	AAAAA	ENV		Cattle isolates in UK; traced to West Africa, 1977/78	11
60	AAAAA	ENV		Elephant isolate, Namibia, 1983	20
64	AAAAA	ENV	STI[e]	Russian vaccine strain	15
129	AAAAA	ENV		Mineral sample, 1972 pXO1-, pXO2-	10
149	AAAAA	ENV		Namibia, 1988; γ -	
152	AAAAA	ENV		Giraffe isolate, Namibia, 1988	
230	AAAAA	ENV		Contaminated soil, Devon, 1991	
234	AAAAA	ENV		Soil isolate taken from sewage spread site, 1992	
240	AAAAA	ENV		Mouse re-isolate of an isolate taken from the same location as ASC 285, 1992	
				Contaminated soil, Zambia, 1991[f]	
264	AAAAA	ENV		Wool isolate, China	
273	AAAAA	ENV		Long hair cow, China	
274	AAAAA	ENV		Horse hair lagging, Kings Cross Station, UK, 1992	
285	AAAAA	ENV		Tannery dump site, UK, 1988	
79	ABAAA	ENV1		Hippo samples, Zambia, 1988	19
91, 93	ABAAA	ENV1		Cow, South Africa, 1937	17
1	AAAAC	VAX	Sterne[g] (NCTC 8234)		
97	CAAEC	VAX	Sterne	Guinea pig re-isolate of Wellcome vaccine strain	
126	AAAAC	VAX		pXO1⁻, pXO2⁻; sewage isolates, UK, 1989	22
183	AAAAC	VAX		pXO1⁻, pXO2⁺, 1976; P⁺, γ⁻	
245	AAAAC	VAX	Sterne	Central Veterinary Laboratory, Weybridge, 1951	
MS191	HAAGC	VAX	Sterne	CAMR vaccine master seed, 1991; derivative of ASC 245	
MS291	AAAAC	VAX	Sterne	CAMR vaccine master seed, 1991; derivative of ASC 245	
WELL	AAAAC	VAX	Sterne	Wellcome vaccine strain	17
68	DAAAC	VAX	Ames	Cow, USA, 1980	21
162, 167	AAAAC	VAX	Ames	Guinea pig re-isolates of ASC 68	21
BA42A	DAAAC	VAX	Ames	Guinea pig re-isolate of ASC 68	
BA40D	AAAAC	VAX	Ames	Guinea pig re-isolate of ASC 68	
2	AAAAB	VOL	Vollum	Gruinard Island, 1950	
3	EAAAB	VOL	Vollum	ASC 6 re-isolate from monkey, 1949	16
6	CAAEB	VOL	Vollum (NCTC 10340)	Cow, UK, pre-1939	
11	AAAAB	VOL	(NCTC 5444)	UK, 1938	
43	AAAAB	VOL	Vollum	ASC 3 passaged 3 times in rats and 5 times in rabbits	
69	GADFB	VOL	New	Human, USA, 1957	13
184	AAAAB	VOL	Vollum[h]	pXO1⁺, pXO2⁻	
185	AAAAB	VOL	Vollum[h]	pXO1⁻, pX02⁺	
328	AAAAB	VOL	Vollum[h]	Progenitor of ASC 184 and 185	
319	AAAAB	VOL		Cow, Ardgay, UK, 1993	
65	BABBD	BRZ		Cow, Brazil, 1982	4
50, 54, 56	BABBE	ZIM		Human epidemic, Zimbabwe, 1982; ASC 54 is γ⁻	6
122,	BABBE	ZIM		pZO1- , PXO2-; sewage isolates, UK, 1989	23
182	BABBF	PAS	Pasteur	France, pre-1880's	12

Other *B. cereus* group strains: *B. cereus* NCTC 2599, NCTC 9939, F4810/72[i], F4433/73, F2532/74, F3484/77, F8035, ASC 112 (designated as *B. cereus* from phenotypic testing); *B. mycoides* NCTC 09680; *B. thuringiensis* HD37[j], HD102, HD225, F2113/78 (Entomocidus).

[a]ASC, Anthrax Section Culture, Research Division, CAMR, Porton Down, Salisbury, England. bFingerprint codes for enzyme/probe combinations are presented in the following order: *Eco*RI/fragment 16, *Eco*RI/fragment SP, *Eco*RI fragment 23, *Hind*III/fragment 16/SP, *Hind*III/fragment 23.
[c]ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures. [d]Unless otherwise indicated, all *B. anthracis* strains are pXO1⁺ pXO2⁺, are susceptible to lysis by gamma phage (γ⁺),and are penicillin sensitive (P[s]) eStrain STI : Sanitary Technical Institute, Peoples Institute of Vaccines and Sera, 380042 Tbilisi, Gotua Street,Georgia. fFound to be resistant to the CAMR diagnostic γ phage but sensitive to a phage isolated from a *B. anthracis* strain from the Etosha National Park, Namibia. [g]Original Sterne Vaccine strain. [h]From Medical Countermeasures/Microbiology, Chemical and Biological Defence Establishment, Porton Down, Salisbury, England. [i]from Food Hygiene Laboratory, Central Public Health Laboratory, Colindale, London, England. [j] HD , kindly supplied by Dr H.T.

DNA fingerprinting of *Bacillus anthracis* strains

G. PATRA[1,2], P. SYLVESTRE[2], V. RAMISSE[2], J. THERASSE[2] and J.-L. GUESDON[1].

[1]Laboratoire de Prédéveloppement des Sondes, Institut Pasteur, 75724 Paris Cedex 15, France, and [2]Laboratoire des Biotechnologies, Centre d'Etudes du Bouchet, B.P. N°3, 91710 Vert-le-Petit, France

Summary and introduction

Strain fingerprinting by ribotyping uses variations in the chromosomal positions of ribosomal RNA (rRNA) genes to identify or group isolates of particular species within a genus. The number of rrn loci varies considerably in bacteria, from a single copy in some species to as many as ten copies in *Bacillus*[1]. The aim of this work was to compare the efficiency of 16S or 23S rDNA probes used for typing *Bacillus anthracis* strains. DNA from avirulent and virulent *Bacillus anthracis* strains and closely related *Bacillus* species strains were cleaved by different restriction endonucleases, electrophoresed on agarose gels, blotted to nylon filters, and probed with appropriately labelled rDNA probes synthesized by PCR[2].

Materials and methods

DNA from attenuated strains and virulent isolates provided by various French culture collections were used for a ribotyping analysis. DNA was amplified using PCR from the 16S and 23S genes of *B. anthracis* to give the probes 16S-493 and 23S-508 respectively. The probes were either non-radioactively labelled using acetylaminofluorene[2] or digoxygenin, alternatively were labelled with [α-^{32}P] dCTP using a ramdom priming method. To avoid descrepancies in the hybidisation patterns that depend on the method of DNA preparation[3], all genomic DNA was extracted using a unique protocol[4], which has been adapted from Henderson *et al*[5] by including the use of CTAB (cetyltrimethylammioium bromide) to remove the polysaccharide-like material.

Results and discussion

From a total of 28 restriction endonucleases tested in preliminary experiments to determine the size of fragments produced, 10 endonucleases were retained : *Acc*I, *Cla*I, *Dra*I, *Dde*I, *Eco*RI, *Eco*RV, *Hae*III, *Hinf*I, *Hind*III, *Sau*3A. These enzymes were used for ribotyping experiments with the 16S-493 and 23S-508 probes. The best results were obtained by digesting genomic DNA with *Acc*I, *Dra*I, *Eco*R I and *Hind*III (Fig. 1). For 18 different *Bacillus anthracis* strains, 4, 3, 4, 5 bands were detected respectively with these enzymes. With *Dra*I, the fragments size ranged from 2.3 to 4.4 kb and from 1.9 to 4.4 kb using the 16S-493 fragment and the 23S-508 fragment respectively as the probe. The most informative patterns and best bands resolution were obtained with the 23S-508 probe.

To overcome disadvantages of radionucleide labelling probes, acetylaminofluorene or digoxigenin-labelling was used. These probes provided sharper bands than those obtained when ^{32}P labelled probes were used. Moreover, because we constructed these rDNA probes from *Bacillus anthracis* template DNA, high stringency hybridisation conditions could be employed.

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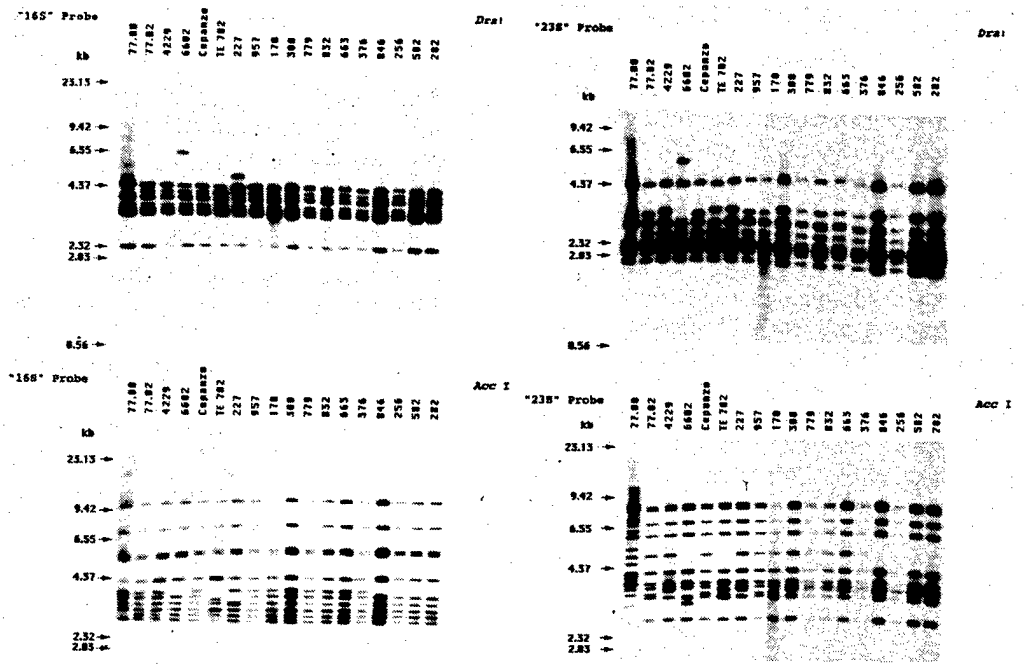

Figure 1. Southern blot of genomic DNA of avirulent and virulent *Bacillus anthracis* strains cleaved by *Dra*I (upper part) or *Acc*I (lower part) and probed with ^{32}P lablelled 16S-493 (left) or 23S-508 (right) rDNA sequences. The *Bacillus anthracis* strains: 77.00, 77.02, 4229, 6602, Cepanzo and 957 were pXO1$^-$ or/and pXO2$^-$. The *Bacillus anthracis* strains: 227, 170, 300, 779, 932, 663, 376, 846, 256, 582 and 282 were pXO1$^+$ and pXO2$^+$.

Phage from different strains of *Bacillus anthracis*

C. REDMOND, I. HENDERSON, P.C.B. TURNBULL and J. BOWEN

Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, UK.

Summary

The lysis patterns of the diagnostic gamma phage and 18 natural or laboratory-induced lysogenic bacteriophages of various *Bacillus anthracis* isolates, were studied on 87 strains of *B. anthracis*, 9 *B. cereus* strains and 41 strains encompassing 22 other *Bacillus* species. While the large majority (85 %) of *B. anthracis* strains were lysed by all the phages and nearly all other *Bacillus* species (90 %) were unaffected by the phages, the few strains exhibiting variable lysis patterns provided evidence of some, possibly phenotypic, difference between the phages. By electron microscopy all the phages appeared morphologically identical. Antigenic analysis revealed a strong antigenic relationship between the phages with 2 major bands in common to all but one phage and minor bands in varying patterns recognized in Western blotting by four different antisera. Restriction enzyme digestion analysis on the phage DNA using a number of enzymes revealed a highly conserved pattern with differences amounting to double and single band shifts. Approximately 80 % of the genome appeared to be shared by all the phages; the remaining 20 % representing a variable region that can differ in size between phages by as much as 15 kb. The genomic digestion patterns were unchanged by passage through a heterologous host indicating that the patterns were phage specific and are not influenced by the host. Nevertheless, phage DNA groups based on these patterns seem to provide a useful way of grouping the host strains from which they were originally isolated and may represent tools for strain differentiation on this basis.

Introduction

This study arose as the byproduct of an examination of lysogenic phages from *B. anthracis* isolates with widely varying histories for their possible value in strain differentiation for anthrax epidemiology.

Description of anthrax phages date from as early as 1931[5].In 1951[8,9], McCloy described a phage designated phage W from strain W bacillus which she believed to be an atypical *B. cereus* or unusual *B. anthracis* strain. The gamma phage was first reported in 1955[2] as a "new variant isolated from the original strain W bacteriophage".

A few reports exist[1, 3, 10, 11, 12, 13, 15, 16] of studies on the gamma phage and other phages capable of lysing *B. anthracis* but these were not concerned with sub-typing the species.

In the early 1960's, Buck et al[4], using 25 phages isolated from 139 cultures obtained from 18 laboratories throughout the world, were able to divide the strains of *B. anthracis* into three groups: those lysed by all the phages (227 strains), those resistant to all the phages (7 strains), and those showing varying lysis patterns with the different phages (30 strains). Of 64 isolates of other *Bacillus* species, lysis was produced in one *B. megaterium* and one *B. subtilis* strain. Cross-neutralisation tests with anti-sera to the phages showed a strong antigenic relationship between all the phages.

The unusually conserved nature of *B. anthracis*, wherever it is found, has long defied attempts to devise strain typing systems for epidemiological and other purposes[6,7]. The observation of the spontaneous appearance of plaques in many of our cultures, together with the availability of new technologies, prompted a re-examination of the possibility that the phages might yet lend themselves to strain differentiation.

Materials and methods

Bacteriophages

The sources of the phages are summarised in Table 1.

The *B. anthracis* cultures were mostly chosen on the basis of having exhibited spontaneous plaque formation. In each case a loopful of culture, with the loop streaked directly through plaques, was subcultured on a horse blood (BA) agar plates which was incubated overnight at 37 °C and examined for spontaneous lysis. With four cultures, where no lysis was found, phage production was induced using mitomycin C (0.45 µg/ml) incorporated in L-agar plates.

Table 1 Sources of phages

Phage	Source
1	Sterne, vaccine strain, cow South Africa, 1937
6	Vollum, Type strain, cow Oxford, pre-WW2
11	NCTC 5444, Fleming, St Mary's, 1938
2nd	Etosha National Park, Namibia, Zebra, 1983
55	Zimbabwe, Human isolate, 1982 (Z7)
122	Untreated sewage sludge sample from Gresford sewage treatment works, cap -ve; Wales; non-capsulating
126	- ditto -
127	- ditto -
134	Zambia, Luangwa Cape buffalo, 1989
201	Etosha National Park, Nambia, Zebra, 1989
203	Etosha National Park, Namibia, Wildebeest, 1991
208	Kruger National Park, South Africa, species unknown, 1991
269	Bovine burial site ± 1942, isolated 1992
310A	Saudi Arabia, child endocarditis 1989; non-capsulating
321	Chinese goat hair, 1993
323	Camel hair, 1993
345	Cheltenham, abattoir site soil sample 1993; non-capsulating
363	Etosha National Park soil sample from site of a carcass (species unknown), 1992
381	Ames (USA) challenge re-isolate (UK) from pig
Z6	Zimbabwe, Human case 1982, diagnostic phage negative
L9	Tannery dump, 1995
Reading bonemeal	Bonemeal, 1995
Diagnostic	Central Vet Lab,Weybridge 1981
16.4.85	Diagnostic phage obtained from Guildford PHL

A number of media, methods and approaches were used for amplifying the phages, including growth in semi-solid L-agar (LA) cultures of *B. anthracis* or in lawns of *B. anthracis* on BA or LA transferred to log-phase cultures of the same host *B. anthracis* in brain-heart infusion broth (BHIB) or L-broth. All methods were effective but the one adopted for most of the project involved repetitive addition of 100 µl of phage preparation to 10 ml of a log phase host culture in BHIB followed by incubation at ≥4 h on a rotary shaker at 37 °C and filtration through a 0.45 µm membrane filter until an adequate titre was achieved. Finally, 1 ml of filtrate was added to a 100 ml log phase culture, again incubated on the shaker for ≥4 h followed by centrifugation and filtration (0.2 µm).

To determine phage titres, lawns of host strains were prepared by spreading 200 µl of log phase cultures on BA or LA plates. After 1 h at 37 °C, 20 µl volumes of 10-fold dilutions (to 10^{-8}) were dropped gridwise onto the lawns. Plaques were counted after overnight incubation at 37 °C.

Host range analysis

The lysis characteristics of the phages were tested on 87 strains of *B. anthracis*, 9 *B. cereus*, 3 *B. thuringiensis* and 38 other strains representing 21 other *Bacillus* species, by placing 15 µl drops of phage preparation gridwise on lawns of *Bacillus* strains prepared as above. Lysis after overnight incubation at 37 °C was scored on a 1-5+ basis, 1+ representing an indentation in the bacterial lawn of growth and 5+ representing a completely lysed zone.

Morphological and serological analysis

Caesium chloride (7.5 g) was dissolved in 10 ml of phage preparation and ultracentrifuged at 30 000 rev/min for 36 h. Fractions (0.5 ml) were removed by means of hypodermic needle and syringe inserted through the top of the tube. The peak active fractions were identified by titration as described previously and were pooled and dialysed in phosphate buffered saline (PBS) with 1 buffer change. After dialysis, the preparations were filtered (0.45 µm), titrated and stored in sterile bijous at 4 °C.

A proportion of the phages were examined under the electron microscope by Mr B. Dowsett (CAMR) and a further set by the NMRI (Naval Medical Research Institute, Bethesda, Maryland, USA) using standard negative staining procedures.

Antisera were raised in guinea pigs to the purified preparations of 2nd, 11M, L9 and "diagnostic" phages by seven intramuscular doses of increasing protein content from 50 µg in the initial dose to 100 µg in the final dose; with Ribi (Tre-Mix) adjuvant (Ribi ImmunoChem Research, Inc., Hamilton, Montana, USA) for doses 1 and 2 and $Al(OH)_3$ (25 % final concentration) as the adjuvant in dose 3 onwards. The anti-sera were harvested 14 days after the final dose.

The anti-sera were used for analysis of serological cross-reactions between the different phages by Western blotting.

Isolation of bacteriophage nucleic acid

Bacteriophage DNA was isolated by a modification of the method of Santos[14].

DNA was isolated from 45 ml volumes of the phage preparations. Bacterial nucleic acids were removed by digestion with 20 µl of DNAse I and 20 µl of RNAse I for 1 h at 37 °C. The phages were precipitated by the addition of sterile zinc chloride (39 mM final concentration) with incubation at 37 °C for 5 min. After centrifugation at 5000 rev/min for 5 min, the supernatants were removed and the pellets re-suspended in 0.5 ml volumes of pre-warmed (60 °C) TES buffer (0.1 M Tris-HCl, pH 8.0; 0.1 M EDTA [disodium salt]; 0.3 % (w/v) SDS) and held at 60 °C for 15 min. After cooling on ice, sterile potassium acetate solution (pH 5.2) was added to a final concentration of 170 mM, and the mixture held on ice for a further 15 min during which a protein precipitate formed. After centrifugation, the supernatant containing the DNA was transferred into 2 separate 50 ml centrifuge tubes and precipitated overnight by the addition of an equal volume of isopropanol and storage on ice at 4 °C. The DNA was centrifuged down and the pellets washed with a minimum volume of 70 % ethanol. After centrifugation, the pellets were dried in a vacuum oven at 40 °C for 30 min and re-suspended
in a minimum volume of TE buffer (10 mM Tris-HCL, pH 8.0; 1 mM EDTA [disodium salt]).

The DNA was further purified by CsCl density gradient centrifugation. Re-suspended DNA (10 ml) was added to 10 g CsCl and mixed. Once the CsCl had dissolved, 100 µl of ethidium bromide was added and, after mixing, the preparation ultracentrifuged at 36 000 rev/min for 48 h.

Under UV illumination, the DNA was removed from the centrifuge tube with a syringe and a needle in a volume of approximately 2 ml. The EtBr was removed from this solution by extraction with CsCl-saturated isoamyl alcohol and the mixture dialysed against TE buffer (10 ml 100x TE in 1 litre sterile distilled water). The optical density of the DNA at 260 nm was determined.

Bacteriophage DNA was digested with restriction enzymes according to the manufacturer's instructions. DNA fragments were analyzed on agarose gels in TBE buffer (EtBr at a final concentration of 0.5 µg/ml), run overnight at 50 V, and photographed with a Polaroid MP-4 Land Camera.

Results

Phenotypic characteristics

The lytic actions of the different phage preparations on the range of *Bacillus* species and strains of *B. anthracis* are summarized in Table 2.

Table 2 Host range analysis of 19 *B. anthracis*-derived phages on *B. anthracis* and other *Bacillus* species.

Lysis zone with all phages tested (0 - 5+ scale)	No (%) of *B. anthracis* strains[a]	No (%) of other *Bacillus* strains
3 - 5+	74 (85.1%)	0
Variable (0 - 5+)	10 (11.5%)	5 (10.0%)[†]
Negative	3 (3.4%)	45 (90.0%)

[a] These strains include the *B. anthracis* hosts used to isolate the phages.
[†] All *B. cereus* strains, 4 from related soil samples (strains WW1 - WW4).

Of 87 strains of *B. anthracis* with unrelated histories, 74 were completely or almost completely lysed (3 - 5+) by 19 phages. Only 3 strains were totally resistant to all the phages, while 10 exhibited variable susceptibility. The majority of phage preparations had titres of >5 x 10^8 pfu/ml.

Among the other *Bacillus* species tested, none showed complete (3-5+) susceptibility to the 19 phages. Five *B. cereus* soil isolates, four from the same site, exhibited variable susceptibility to one degree or another to the different phages.

The phages were morphologically indistinguishable by electron microscopy with icosahedral heads and long, sheathless non-contractile tails (Fig. 1), conforming to previous detailed descriptions[15] and to the morphology of type B bacteriophages.

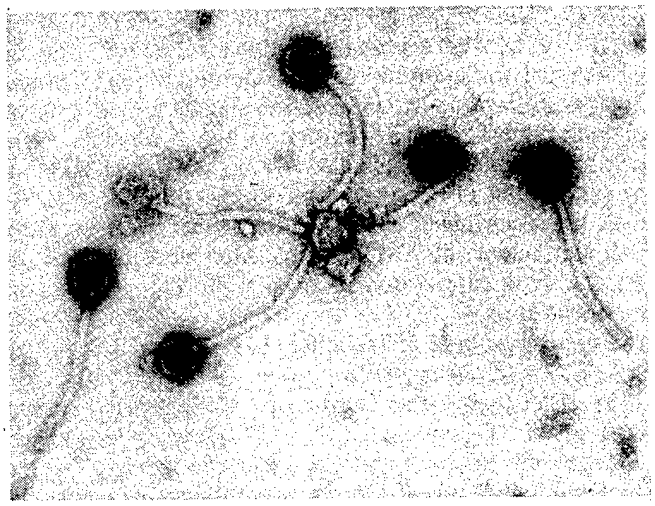

Figure 1. Negatively stained preparation of the diagnostic phage (x 102.960)

Western blotting revealed a strong antigenic relationship between all but one of the phages, whether grown in homologous or heterologous hosts, with two major bands in common to all of them and varying patterns of minor bands recognized by all four anti-sera (Fig. 2). The single exception was phage 208, amplified in its homologous host, which was apparently unrecognized by any of the antisera.

Genotypic characteristics

Restriction digest patterns of the phage suggest that the genome size is approximately 35 kb with a maximum of 45 kb. The difference in genome sizes is suggestive of a variable region within phage genomes. The DNA is resistant to, or does not possess sites for digestion by restriction enzymes from other *Bacillus* species; the exceptions noted were *Bsc* I and *Bcl* I.

Digests of phage DNA with *Eco*R I, *Hae* II, *Hae* III (Fig. 3) and *Hin*d III, resulted in phage specific patterns that were not influenced by which of the hosts was used to amplify each phage. However, the patterns could be grouped and appeared to supply a means of grouping the host strains from which they were originally isolated (Table 3).

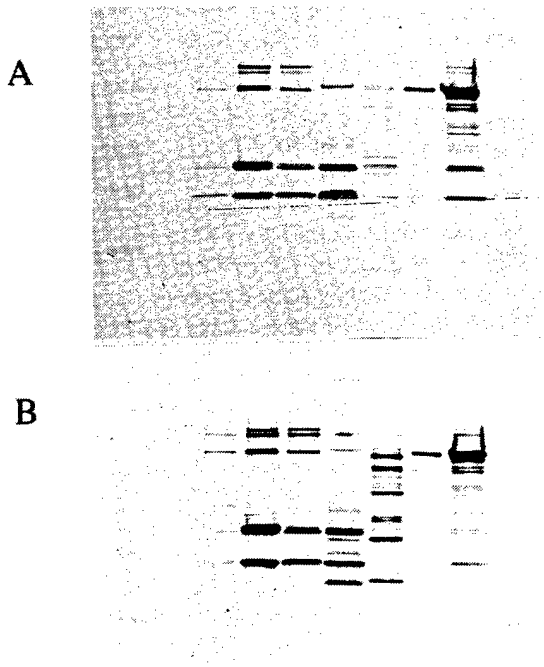

Figure 2. Western blots of seven *Bacillus anthracis* bacteriophages developed using (A) anti-2nd phage serum, and (B) anti-L9 serum

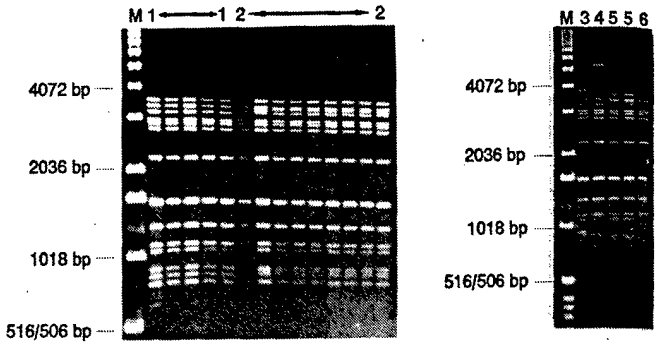

Figure 3. *Hae* III restriction digestion pattern analysis of phage DNA

Table 3 Grouping of *B. anthracis* phages based on restriction enzyme digestion patterns.

Group	Phage	Restriction enzyme/Designesd pattern			
		*Hae*III	*Eco*R I	*Hae*II	*Hind*III
A	55, 122, 126, 203, 208, 269, 310A, 321, 345, 363, 381	HR 1	ER 4	HT 3	HD 2
B	Reading Bonemeal, 323	HR 1	ER 4	HT 4	HD 2
C	134	HR 1	ER 2	HT 3	HD 3
D	2nd	HR 2	ER 3	HT 2	HD 1
E	1	HR 3	ER 1	HT 1	HD 1
F	L9	HR 3	ER 1	HT 1	HD 4
G	6	HR 3	ER 2	HT 1	HD 1
H	127	HR 3	ER 2	HT1	HD 2
I	201, Z6	HR 4	ER 5	HT 5	HD 3
J	W. Weybridge, 10.1.81	HR 5	ND	ND	ND
K	JAC 16.4.85	HR 6	ER 3	HT 6	HD 5

ND = digests not done. *Hae*III pattern type 5 (HR 5) was identified during a previous study; the opportunity to examine the other enzyme patterns is awaited.

Discussion

Host range analysis confirmed the previous conclusions[4] that spontaneously produced phages of *B. anthracis* do not lend themselves to conventional phage typing of this species. The proportions of *B. anthracis* strains found to be sensitive to lysis by all, some or none of the phages, remained very consistent in repeated studies and, remarkably, differed by <1 % from those noted three decades ago[4] with a totally independent set of strains and their phages. The close morphological and antigenic relationships also observed before[4, 15] were similarly confirmed. Western blotting, however, indicated that, despite extensive cross-reactivity, the phages did give different banding patterns, and show different arrangements of the cross-reacting antigens. These patterns were independent of the host in which the phage was amplified and may correspond to the capsid and tail polypeptides described previously[15].

Further work would be needed to establish whether the Western blotting patterns correspond to the DNA digestion patterns. The latter appeared to offer a more manageable tool for practical purposes. All the *B. anthracis* hosts could be placed into a group according to the restriction enzyme digestion patterns of this phage but exactly half of them fell into Group A. Further genetic analysis may improve the scheme, particularly in breaking down the large group.

One of the hardest tasks within the project was amplification to an adequate titre of some of the phages in their homologous hosts. Explanations for this may lie in the laws of lysogeny, although the problem was not encountered with all of the strains. While some of the differences in Western blotting may have been attributable to the degree of amplification achieved, an

explanation for the absence of recognition of phage 208, amplified in its homologous host, by any of the antisera awaits further investigation.

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Bacillus anthracis strain identification by means of phenotypic systematic criteria.

N. A. STARITZYN, N.A. SHISHKOVA, L. I. MARININ and A. V. STEPANOV

State Scientific Center of Applied Microbiology, Obolensk, Russia

It is well known fact that variability is a characteristic feature of the genus *Bacillus*[1,2]. This has resulted in attempts to elucidate the reasons for *B. anthracis* strain diversity on the level of structural and functional peculiarities of its pathogenicity genes, *pag, lef, cya, cap* with plasmid localisation[2,3] and to develop not only an identification system for the genus *Bacillus,* but also an intraspecies system for *Bacillus anthracis* strain identification. While identification of virulent or avirulent strains can be done by microbiological, biochemical or by the genosystematic methods[4-8] (visualisation of capsule in vitro and in vivo, investigation of plasmid profile and their restriction map, DNA hybridisation or PCR reaction), more detailed intraspecies classification of *Bacillus anthracis* strains with intermediate virulence is still needed. For medical and veterinary purposes, a strain identification by phenotypic criteria of virulence and toxity are adequate, but for typing strains for ecological and epidemiological purposes, other characteristics, such as the resistance and stability of the bacteria in the natural environment, are necessary. For this purpose, such parameters as thermoresistance of spores, viability after drying on silica gel, and UV stability can be used[9-12]. The aim of this research was to evaluate extreme values of biological properties of 17 *B. anthracis* strains and to elaborate phenotypic criteria for their identification.

Materials and methods

17 strains from the museum collection of State Centre of Applied Microbiology were used. Spore suspensions were prepared by culture of these strains on undefined beef extract solid media for 4 days at 32°C and suspending spores from the surface of media in 30% glycerol.

Virulence (LD_{50}) was determined by intraperitoneal inoculation of white mice with suspensions containing ca. 10, 50, 250, 1250 spores in 0.5 ml of physiological saline. Toxity was recorded as reciprocal values of cfu of *B. anthracis* in rabbits' blood at the moment of death following subcutaneous injection. Thermoresistance was determined as a percent of spore survival after heat shock of the spore suspension for 10 minutes at 85°C. Viability of the spores was evaluated as a percent of cfu after drying 0.5 ml of spore suspension with 4.5g of silica gel. Spore stability was determined as a percent of cfu after the UV irradiation of spore suspension (30W, 25 cm, 15-60 sec). The borders of grade intervals were determined after evaluating of extreme values of biological properties of the strains studied by means of the Brucs and Karusers, formula:

$$i = \frac{X_{min} - X_{max}}{5 \operatorname{Lg} n}$$

X_{min}, X_{max} - extreme values of biological properties;
n- number of strains studied;
i- quantity of grade intervals

Results and discussion

The extreme values of biological properties found for the 17 strains are summarised in Table 1, and the results of determining of borders of grade intervals in Table 2. Biological properties of *B. anthracis* strains evaluated in balls are given in Table 3.

For the purposes of differentiating intraspecies groups of *B. anthracis* strains according to their pathogenicity and ecological and epidemiological characteristics, five biological properties of each were investigated. In contrast to numerical taxonomy, which utilises approximately 200 tests[13], it seems to be sufficient to evaluate such properties as virulence, toxity, thermoresistance, viability and UV stability, which determine the medical, ecological and epidemiological profile of the strain. The formal designation of biological properties of strains, as given in Table 3, permits the distinction of intraspecies groups. For example, strains with the overall summary of values 13-17 (strains NN 4, 5, 8, 9 13, 14, 17) may be considered as strains with medical, epidemiological and ecological importance. On the other hand, the virulence of such strains as NN 2, 7, 11, 16 amounts to 1 ball only and their role as a causative agent of anthrax is minimal. The remaining strains are represented by intermediate values; the basis of this needs to be investigated. So, the phenotypic systematic criteria permit not only the evaluation of the epidemiological, ecological, medical and veterinary importance of the strains, but also highlight strains which should be further investigated to understand the basis of their variability.

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Table 1. The extreme values of biological properties of *B. anthracis* strains

Biological property	**Extreme value**
Virulence, spores	13-174
Toxity, cfu.ml-1	0.004-16.7
Thermoresistance, %	17-84
Vialability, %	14-90
UV stability, %	14-55

Table 2. Borders of grades intervals of biological properties studied and evaluation of grade intervals

Biol. property	**Borders of grade intervals and their evaluation in balls**			
	1	2	3	4
Virulence	>150	149-45	44-11	<10
Toxity	<0.003	0.004-3.5	3.6-7.1	>7.1
Thermorsist.	<15	16-43	44-71	>72
Viability	<10	11-46	47-82	>83
UV stability	<10	11-27	28-42	>46

Table 3. Evaluation in balls of biological properties of *B. anthracis* strains

Strains	**The values of biological properties**					**Summarised value**
	Virulence	Toxicity	Thermoresistance	Viability	UV Stability	
1	3	2	2	2	3	13
2	1	1	2	3	2	9
3	2	2	3	3	2	12
4	2	2	2	4	3	13
5	2	2	3	4	2	13
6	3	2	2	2	3	12
7	1	2	3	2	3	11
8	3	2	3	2	3	13
9	3	4	2	2	3	14
10	3	2	2	2	2	11
11	1	1	2	2	3	10
12	2	2	2	3	3	12
13	3	2	3	3	2	13
14	2	2	2	3	4	13
15	3	2	2	3	2	12
16	1	2	3	2	2	10
17	3	2	4	4	4	17

Risk assessment of former tannery sites

R BÖHM and JULIA OTTO

Institute für Unwelt- and Tierhygiene Sowie Tiermedizin mit Tierklinik der Universität Hohenheim, D-70593, Stuttgart, Germany

Introduction

Former tannery sites are known to represent a certain risk with respect to anthrax spores, heavy metals and organic pollutants. No systematic scientific investigation has been done in Germany until now, either to determine the likelihood of disturbing viable anthrax spores remaining in the soil at these sites, or to determine which factors may influence the actual risk of infection for humans and susceptible animals. The aim of this investigation was to come to a preliminary risk assessment, by systematic sampling of soil from different tannery sites, in a town representative for leather industry in the first half of this century. In the past, several cases of anthrax in tannery workers and in cattle grazing close to wastewater receiving creeks had been reported to the Authorities. Therefore, it is likely that hides contaminated with spores of *Bacillus anthracis* had been processed in most of the factories investigated.

Materials and methods

Samples were taken from the soil at several depths, depending on the situation and the intended aim. From core samples, a visual examination was used to determine those parts of the soil columns which indicated the presence either of organic materials, like leather, hair and slurry, or of soil strata generated by the application of organic sludge and wastewater. These were examined for the occurrence of spores of *Bacillus anthracis.*

A summary of data from the tanneries is given in Table 1. The method used for the detection of anthrax spores is described elsewhere by Beyer *et al* (1995). A total of 330 samples from different tannery sites was analysed. They originated from parts of the mills where the raw hides had been treated, where the tanning equipment had been located, where leather wastes had been discarded,from wastewater pipes, pits, and treatment plants, from places where the sewage sludge was stored and disposed, from waste water irrigation areas and from the banks of the waste water receiving river.

Results

In most of the samples investigated, no viable spores of *Bacillus anthracis* could be found (detection limit less than 5 spores/100 g). The results are summarised in Table 2. Most positive samples could be found in one tannery which closed shortly a fter some spectacular cases of human anthrax. Here, virulent spores could be found at places where the raw hides had been stored and treated, as well as in the waste water system.

The other locations where positive samples could be found were an area where tannery and skin-wool offal had been disposed and soil from the banks of the former receiving water area, in the layer above the peat stratum.

Since the sampling density was relatively low in the irrigation and waste disposal areas, no final conclusions could be made. However, the results support the following risk assessment:

All areas where raw hides had been handled and processed carry an increased risk for the occurrence of spores of *Bacillus anthracis*. The risk seems to be greater if a factory ceased operation shortly after an anthrax event.

All places where raw hides and wool-wastes had been disposed of are at risk.

A soil close to meandering creeks and rivers is at risk, both in and below the layer of ancient sediments.

A risk of infection therefore exists for humans and more so for susceptible animals at the time earth is being moved in high risk areas. As long as the soil remains undisturbed, the risk both for humans and for animals can be considered to be extremely low.

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Table 1. Description of the locations where samples had been taken at former Tannery-Sites.

Description of Tanneries			Samples taken from									
Tannery No	Anthrax cases reported	Dry hides processed	Raw material processing	Tanning workshop	Leather waste	Sewage sludge	Sludge lagoons	Irrigation fields	Waste water pipes	Receiving water	Hide storehouse	Sewage treatment plant
1	Yes	Yes	-	-	-	-	-	+	-	-	-	-
2	Yes	Yes	+	+	-	-	+	+	+	-	+	+
3	Yes	Yes	+	+	+	+	+	+	+	+	+	-
4	Yes	Yes	+	+	-	-	+	+	+	-	-	+

Table 2. Number of samples where *Bacillus anthracis* could be detected by PCR at the investigated Tannery sites (positive/total samples).

Tannery No	Raw material processing	Tanning workshop	Hide store house	Sewage treatment plant	Leather waste	Sewage sludge	Sludge lagoons	irrigation fields	Waste water pipes	Receiving water	Colouring workshop	Receiving water river banks
1				0/6				0/118				
2	0/9	0/12	0/2					0/6	0/1			
3	0/4	0/2	0/5		2/35	0/4	0/1	0/2	0/2	0/12		
4	1/25			4/8			0/2	0/9	1/9			
Other							0/3				0/12	2/15

Workplace health hazards from anthrax-contaminated textiles

B. CROOK[1], B. HOULT[2] and A.C. REDMAYNE[1]

[1]*Biomedical Sciences Group, Health and Safety Laboratory, Sheffield S3 7HQ, UK.*
[2]*Field Operations Division, Health and Safety Executive, Horsforth, Leeds LS18 5TW, UK.*

Introduction

Animal fibres, such as cashmere goat hair, may be contaminated by anthrax spores if they originate from areas where anthrax is endemic in the animal population. Workers handling the contaminated material in the textile industry therefore are at risk of contracting either cutaneous or inhalation anthrax, unless preventive measures are taken. "Woolsorter's disease" (a local name for inhalation anthrax) claimed many lives in Yorkshire earlier this century and led to the adoption of a number of legal measures aimed at reducing the risk. Cases of human anthrax in the UK are now very rare and the last reported fatality from the disease was in 1974. The hazard to health, however, is still present and it is necessary to ensure that the risk of contracting the disease is kept at a very low level. The continuing existence of the hazard was illustrated in Switzerland between 1978 to 1980 when 24 workers in a textile factory handling goat hair from Pakistan contracted cutaneous anthrax and one had inhalation anthrax[1,2].

The greatest risk of exposure to spores comes when the unprocessed fibre is being sorted by hand (a highly skilled operation), or during the earlier processing stages such as blending, carding or combing. Further into the processing, the source of potentially contaminated material (dust, animal dander, fats, lanolin or blood clots in the hair) may be removed by handling and washing (scouring).

The principal risk reduction measure which can be applied to animal fibres is to process only raw material which is free from infection. This can be achieved by acquiring the raw material from a country which is known to be free from anthrax and/or from animals which have a veterinary certificate declaring them to be free from infection. However, some of the major sources of goat hair (e.g., cashmere) are in countries in which anthrax is endemic and freedom from anthrax spores for this material can only be achieved by a reliable process of disinfection, supported by an appropriate method for sampling and analysis.

In the UK, the original disinfection process using formaldehyde was first developed in 1918[3]. Since then, methods have changed and it has been found necessary to conduct further work to look at existing disinfection methods, and to investigate new methods both of disinfection and sampling/analysis.

Disinfection methods

Recent experiments have been undertaken using *Bacillus cereus* NCTC 2599 spores as surrogates for *B. anthracis*. Initially we examined the effects at 43°C over time of formaldehyde at concentrations of 2%, 1.2% and 0.9%. Our results are shown in Fig. 1. Other factors, such as heat (for drying the treated fibre) and the continuing presence of formaldehyde after treatment may also have an important influence on the results obtained by the actual disinfection process applied by the scourer. Heat treatment alone at 70, 90 and 100°C had little effect on numbers of viable spores over a 30 min period, but a combination of exposure to 0.9% formaldehyde followed by heat treatment brought about an improvement in the kill rate (Fig. 2). However, it is evident that the effectiveness of this balance of heat and disinfectant treatment is fragile. Further work is in progress to examine the potential protective effect on spores of organic material and animal fibre.

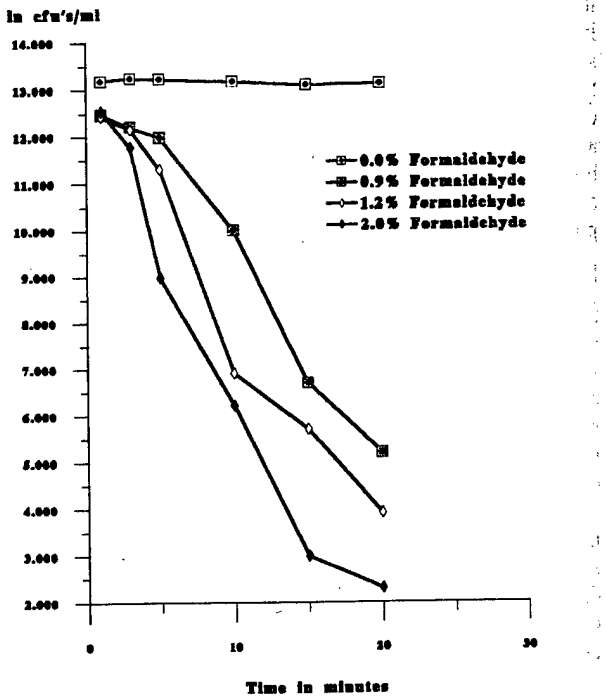

Fig 1. Rate of kill of formaldehyde at three concentrations on *Bacillus cereus* spores.

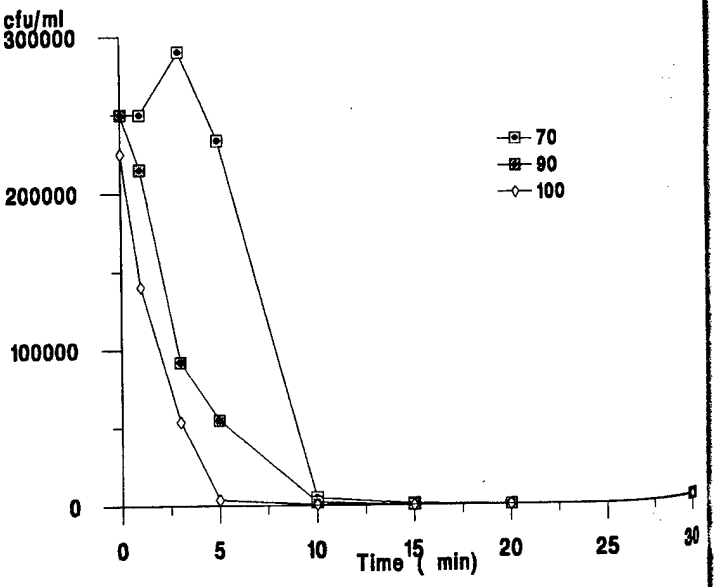

Fig 2. Effect on *B. cereus* spores of a combination of 0.9% formaldehyde treatment followed by heating to 70, 90 and 100°C.

Sampling and analysis

When there is a need for microbiological analysis of imported goat hair, random grab samples are taken for analysis

from quarantined bales. How representative of the bale these may be is open to debate. A typical bale may be 1.0 x 0.75 x 1.2 m in size and weigh 300 kg, comprising hair from many animals, only some of which may support anthrax spores. If, for example, a 10 g portion of the goat hair is analysed, it represents only 1/30,000th of the whole bale. This underlines the importance of taking a statistically relevant number of samples that will be representative of the whole bale, perhaps following sampling procedures such as those devised for testing other constituents in wool[4].

Conventional microbiological analysis is undertaken by preparing pour plates from an aqueous suspension of the sample, counting colonies and using confirmatory tests on colonies suggestive of *Bacillus anthracis*[5], but it can take several days. For imported textiles, the material under test must remain in quarantine until testing is complete, with cost implications for the importer. The test relies on culturability of the spores, which assumes efficient dormancy breaking of spores under *in vitro* conditions and depends to some extent on skilled subjective observation and recognition of "typical" colony characteristics. Recent advances in detection and analytical methods, e.g., molecular techniques, may offer a more rapid and objective analytical tool. Recent studies[6,7,8,9] have reported the development of gene probes with high specificity for *B. anthracis* when tested against other bacteria, including other *Bacillus* spp. They are also able to differentiate between virulent and avirulent environmental strains without culturing. Coupled with polymerase chain reaction (PCR) to amplify the signal, this could provide a rapid and sensitive analytical method, which may also allow more samples to be taken for analysis, to make an evaluation of the microbiological quality of a batch of goat hair more representative.

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Literature on anthrax as a biological weapon

J. MIERZEJEWSKI and M. BARTOSZCZE

Military Institute of Hygiene and Epidemiology, 2 Lubelska str., 24-100 Pulawy, Poland

Anthrax spores have been studied as a candidate biological weapon and are considered almost equal in weapon potential as botulinum toxin. Published fragments of scientific programmes show that these studies were carried out in the former USSR, Japan, UK,USA and more recently in Iraq.

The end of the cold war and the reduction of armed conflicts have led to a general reduction in weapons of all kinds including biological weapons.

Spores of anthrax are of particular importance as a weapon because of their innate resistance. The eradication of the stockpiles of this weapon may be only part of a general disarmament problem.

The eradication of the agent from the environment following weapon evaluation trials pose a particular problem. The disinfection of Gruinard island is characteristic. The treatment of an area of four hectares of the island required considerable manpower, machinery and an approved method of treatment (280 tonnes of 40% formaldehyde, 2000 tonnes of sea-water, 50 kilometres of irrigation tubing and a decontamination team over a six-week period). This was followed by a further period of evaluation of the success of the treatment including sheep grazing as a demonstration of confidence [5].

There is no doubt that many other similarly contaminated sites exist. Nothing is known about the contamination state of soils on Japanese trial areas (Beiyinke, Pin Fan,Chanchun,Nankin, Anda and others[2]). The same is true of Sverdlovsk and its environs in the former USSR where livestock died of anthrax infection[6] and other areas of this country where similar studies were conducted[4]. There is no data on experimental ranges in the USA where biological weapons trials were carried out[1]. The ecological consequences of Iraqi trials are unknown [3,7]. It is possible that these trial sites, which may be polluted with huge numbers of anthrax spores, could become a delayed action bacteriological bomb which could act as a source of future epidemics and epizootics similar to those in ancient and medieval times. This might be even more likely if human and animal immunity decreases as a consequence of wars, hunger and ecological catastrophes.

Also, manufacturing sites producing anthrax weapons, vaccines and anthrax antisera require hazard assessment. Laboratory animals are often used for production and evaluation of the effectiveness of weapon agents. Very often the remains of infected animals were buried without incineration assuming that anthrax spores would not survive for long periods. It is now known that these spores will remain viable for decades, as on Gruinard Island, or for centuries (Vos, our own report).

There are inadequate or non existent records of burial grounds for animals that have died of anthrax and this can lead to the accidental exhumation of the remains of animals containing viable anthrax spores during such activities as excavation and building work.

Questions and conclusions

1. Excessive deposition of anthrax spores in discrete sites due to bomb and missile detonations, aerosol spread, mass burial of anthrax contaminated animal carcases, may constitute delayed action biological time bombs.

2. Such a time bomb may represent a hazard for future generations of humans and animals especially if their immune systems are adversely affected by war, hunger or other catastrophes.
3. The number of other sites similar to Gruinard Island should be revealed and mapped so that future generations can be forewarned.
4. National health authorities of the countries involved and the WHO should give appropriate advice to inhabitants in the locality of these anthrax contaminated sites.
5. If the decontamination method used on Gruinard Island is considered as a model scheme it should be tried on contaminated sites elsewhere.
6. A future anthrax workshop should consider the problem of contaminated sites.

We address the above recommendations on this important problem to workshop participants particularly those on the WHO Anthrax Working Group. The UK method for decontamination of anthrax weapons trials areas should be encouraged. We wish to encourage the organizers of subsequent workshops to include this problem in the programme.

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Biological and toxin weapons: strengthening the arms control regime

A. P. PHILLIPS[1] and M. R. DANDO[2]

[1]Chemical and Biological Defence Establishment, Porton Down, Salisbury, Wiltshire, SP4 0JQ, [2]Department of Peace Studies, University of Bradford, Bradford, West Yorkshire, BD7 1DP, UK

The defence literature increasingly contains articles indicating official concern over the possible development and use of biological weapons[1,2]. Such concern is reasonable in the light of the devastation that could follow the effective use of even a few kilograms of an organism such as anthrax3, suggestions that a number of countries could have secret offensive biological weapons programmes today, and the fact that several countries have already produced biological weapons in the second half of the 20th Century[4].

Biological and toxin weapons are already outlawed by the 1972 Biological and Toxin Weapons Convention (BTWC) which now has over 130 State Parties. Unfortunately, in the political climate prevailing when the BTWC was negotiated, it was not possible to agree to any effective verification conditions. However, there is an increasing measure of agreement between States Parties that the BTWC should be strengthened by the addition of compliance measures, to further discourage the development and use of such terrible weapons.

The current initiative towards strengthening the BTWC is the result of many years effort. It also parallels progress in arms control measures for other weapons of mass destruction. The Nuclear Non-Proliferation Treaty has just been given an indefinite extension and, despite some current testing, probably will soon be followed by a Comprehensive Test Ban Treaty. Together with the ongoing US and Russian strategic arms reductions, this could signal the eventual end of nuclear weapons proliferation. Similarly, the Chemical Weapons Convention (CWC) which is likely to come into force next year will oversee the destruction of existing CW arsenals, and its system of declarations and inspections will result in a degree of scrutiny of industrial scale chemical production world-wide that will deter the potential proliferator[5]. The CWC overlaps with the BTWC because both prohibit the acquisition of toxins as weapons.

The BTWC lacks any organisational resources between its five-yearly review conferences. These reviews do, however, provide the possibility of flexible development of the Convention to meet changing circumstances. Thus, in 1986 and 1991, certain confidence building measures (CBMs) were agreed, in the form of annual data exchanges. The present list of CBMs includes provision of information on high containment facilities, unusual outbreaks of disease, legislation and regulations related to implementation of the Convention and export and import of microorganisms, past offensive or defensive biological research and development programmes, and vaccine production facilities. Unfortunately, the CBMs could only be made politically binding rather than legally binding, and only a minority of States have complied properly with these requirements.

Additionally, at the Third Review Conference in 1991, it was agreed that Government experts should examine potential verification measures for the Convention. At the 'VEREX' series of meetings over the next two years, 21 potential verification measures were evaluated.

A Special Conference of the State Parties held in September 1994 reviewed the VEREX work and accepted the VEREX view that some combination of measures, including both off-site and on-site measures, could be useful for the main objective of the Convention. The Conference then mandated the further meetings (a new Ad Hoc Group) that are now underway. As well as a system of compliance measures, the current round of meetings may also result in suggestions for further development of the present conference building measures, measures for the investigation of alleged use of biological weapons and proposals for enhanced exchange of technology and cooperation in the peaceful uses of microorganisms and toxins.

It would be unwise to underestimate the difficulties involved in agreeing an effective, legally binding system of compliance monitoring for the BTWC. There are some States which have doubts about the extent to which the Convention is effectively verifiable, particularly in view of the fact that valuable, commercially confident information and national security information must be safeguarded during intrusive inspections.

There are also continuing problems over the differing perceptions of the extent to which technological cooperation should be carried out within the Convention. Outside of the Convention, but obviously related to its purpose, the export licensing arrangements of some countries, and particularly the arrangements that are harmonised within the Australia Group of industrialised countries, have been attacked by a minority of States Parties. The Australia Group countries originally started work together in order to restrict known proliferation of chemical weapons, but recently they have extended their agreements to cover lists of pathogens and toxins and certain types of biological production and handling equipment.

On the other hand, it does appear that many States Parties can see the value and potential effectiveness of a comparatively simple and low-cost compliance monitoring regime operated under the BTWC. Such a regime would require mandatory declarations of facilities whose capabilities have a high relevance to the provisions of the Convention. The declarations would be backed up by provision for various categories of inspections, eg. informational visits at declared facilities in circumstances when there are no prior compliance concerns, and challenge inspections at declared or undeclared facilities at the request of another States Party. These visits/inspections would differ from the health and safety or quality assurance type of inspection performed by national regulatory bodies in that there would be no automatic access to confidential information. The managed access procedures pioneered in the UK in the developmental stages of the CWC in order to protect sensitive information have also been tried out in practice inspections in a BTWC setting, and have been found to equally relevant. Many argue that arrangements to safeguard confidential information would work best if the BTWC has a professional, career inspectorate, rather than ad hoc inspectors drawn from academe or industry, and that this will also provide the best framework for competence and impartiality.

Given the amount of work that has already been carried out at the VEREX meetings, it is not impossible that a BTWC protocol could be drafted in time for presentation at the Fourth Review Conference late in 1996. If this is not achieved, the mandate from the 1994 Special Conference does allow for a longer period of meetings, and presentation of a protocol to a further Special Conference after 1996.

What, one might ask, has all this to do with biologists going about their peaceful work? It can surely be agreed that we would all like to live in a world where the use of biological weapons remained a very remote possibility, and that a BTWC with rigorous compliance monitoring arrangements would form a vital part of an overall scheme designed by the international community for that purpose. If that is accepted, then biologists might take account of the fact that the Chemical Weapons Convention looked a very remote possibility for many years, but was nevertheless agreed - in no small measure due to the constructive efforts of chemists in the chemical industry who are anxious that their technologies should not be misused.

Biologists likewise can play a constructive role in the moves towards strengthening the BTWC, for example by making sure that they and their professional associations track the progress of the negotiations over the next few years so that they may seek to influence the process if they feel they can add something useful. Another crucial role for biologists lies in using their expertise to ensure that there is widespread public understanding of the ethical responsibilities of the scientist and what is at stake if this knowledge and technology are misused, and thus the importance of establishing international mechanisms to increase the transparency of work with pathogens and toxins in order to establish, in the words of Article I of the Convention, its 'justification for prophylactic, protective or other peaceful purposes.' Effective implementation of global prohibition regimes requires a broadly based consensus of support[6].

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Inactivation of *Bacillus anthracis* vegetative cells and spores by gamma irradiation

[1]JANE E. BOWEN, [2]R.J. MANCHEE, [2]SHARON WATSON and [1]P.C.B. TURNBULL

[1]Anthrax Section, Centre for Applied Microbiology and Research, and [2]Medical Countermeasures, Chemical and Biological Defence Establishment, Porton Down, Salisbury, Wiltshire, UK

Summary

Traditionally, vegetative bacteria and bacterial spores are killed by either heating or chemical treatment. In the case of *Bacillus anthracis*, these methods are sometimes unreliable and are also unsuitable if antigenic integrity is essential. Inactivation by gamma irradiation may be the appropriate alternative, although long exposures are required to kill spores. In a systematic study using Sterne and Ames strains, vegetative cells and spores required 2.85 and 41.5 kGy respectively for complete inactivation. However, in using these results for guidance when irradiating vegetative and spore suspensions of 31 strains for a client, one vegetative cell preparation containing spores at 0.04% of the total viable count and 7 spore preparations failed their sterility tests following doses of 44 kGy, albeit with very low numbers of spores still viable. Resistance to the killing effect of irradiation would appear to be strain dependant. Electron microscopy indicated that 41.5 kGy produced no obvious morphological changes. Detailed data on antigenic integrity of irradiated spores and cells are not available yet but client permission was obtained to report that they show apparently normal interactions with antibodies to non-irradiated anthrax antigens.

Introduction

The production of antibody reagents as a component in test kits for the rapid detection and identification of potential biological warfare agents is one of the main requirements of defence programmes.

In the case of anthrax, isolation of specific antigens from the spores of *Bacillus anthracis* presents particular problems because treatments needed to render them inactive and safe to handle on the open bench, such as heating at 90°C for 3 h or exposure to 5% formaldehyde for several days, also denature the protein components of the organism and probably damage their antigenic properties. Gamma irradiation disrupts the nucleic acids of the organism with a minimal effect on the protein components.

The primary mode of action of gamma irradiation is considered to be the production of chemically active free radicals which combine with DNA components. Previously it has been shown that vegetative and spores forms of *Bacillus* spp. were inactivated by irradiation and that the spores were at least 10 times more resistant than vegetative cells[1], but no attempt was made to examine the antigenic properties of the inactivated organisms. We have used a ^{60}Co source to good effect to inactivate both the spores and vegetative bacteria of *B. anthracis*.

The morphologies of spores inactivated by gamma irradiation and formaldehyde were compared by electron microscopy.

Methods and materials

Strains for systematic study

Vegetative bacilli of the Sterne and Ames strains of *B. anthracis* were grown in 50 ml batches in conical flasks by overnight incubation at 37°C in an orbital shaker. Total viable counts on nutrient agar were carried out on 5 ml samples of the cultures and a viable spore count was carried out on the same samples after they had been heated at 60°C for 1 h to kill vegetative bacteria.

Spores were produced by culture on sporulation agar in Roux bottles. Incubation was continued until at least 90% of the bacterial population was in the form of spores, as estimated by microscopy, usually after 2-3 days. The spores were harvested from each Roux bottle with 20 ml of sterile deionised water (SDW).

The suspension was centrifuged at 10,000 x g for 10 min; the pellet was resuspended in 20 ml of SDW and left overnight to allow lysis of vegetative forms. The debris was separated from the spores by differential centrifugation.

Strains for practical application

Spores and vegetative cells from 38 isolates in the Anthrax Section culture (ASC) collection, chosen to represent the widest possible isolation history in terms of human, animal, environmental and geographical source, were prepared according to client instructions.

To prepare vegetative forms, colonies from blood agar (BA) purity plates were transferred at the end of the day to fresh BA plates and incubated at 28°C overnight. First thing the next morning, a loopful of growth was transferred to 10 ml of brain-heart infusion broth (BHIB) and this was shaken on a rotary shaker at 37°C for 2 h after which 100 µl volumes were spread on BA plates, 10 per culture. These, in turn, were incubated at 37°C for 6 h. The growth on the plates was harvested with 2 washes of approximately 5 and 3 ml of ice-cold phosphate buffered saline (PBS). The suspensions were kept ice-cold from then on. They were washed 3 times in a refrigerated centrifuge with a final suspension in 5 ml of PBS. Counts and tests for the presence of spores were then done.

Spores were prepared as described above except that the inocula for the Roux bottles came from heat-treated (62.5°C x 15 min) washings of 10 ml sporulation agar slopes which had been incubated for one week at 28°C. The Roux bottles were incubated at 28°C for 10-14 days before final harvest. Spores were checked by phase contrast microscopy.

Irradiation in systematic study

A 4000D ^{60}Co gamma source was supplied by Mainance Ltd. (Waterlooville, Hants, UK). The source activity was approximately 50000 GBq and it delivered a dose of about 900 Gy h^{-1}. The source control equipment automatically compensated for loss in activity due to decay.

The organisms to be irradiated were dispensed in 10 ml volumes into polystyrene universal containers. Spores were suspended at a concentration of $1x10^9$ or $2x10^8$ and vegetative cells at $1x10^7$ viable organisms/ml. The universal containers were placed in specially designed screw-capped metal containers which were then placed in the central chamber of the irradiator pot. The samples were then transported into the core of the irradiator for the time required. Dose levels of up to 45 kGy were administered.

Irradiation for practical application

Approximately 2.5 ml volumes of the vegetative and spore suspensions were put into 4 ml Nalgene (Nalge Co, Rochester, NY) polypropylene bottles. These were packed in SAF-T-PAK 254 jars (Air Sea Containers Ltd., Birkenhead, UK) which were, in turn , packed into SAF-T-CASEs (Air Sea Containers) embedded in dry- ice.

In an unrelated study, 5-6 ml volumes of Ames strain spore suspensions in 4 SS34 centrifuge tubes, in which they had been washed, were similarly packed into SAF-T-PAK 254 jars and SAF-T- CASEs for irradiation. To give added protection during freezing, glycerol was added to the suspension (15% w/v final concentration).

One SAF-T-PAK 254 pot contained two 30 ml Nalgene bottles in which had been placed dosimeters; in one of the bottles, a dosimeter was fully immersed in water.

Irradiation from a ^{60}Co source was carried out at a commercial premises (Isotron plc., Swindon, UK). Doses were assessed by dosimeters on the outside of the SAF-T-CASEs. Vegetative preparations were given doses of 10.6 kGy and spore preparations received 44 kGy.

Viability assays (systematic study)

Following each dose of irradiation, the numbers of organisms surviving were determined after 48 h and then after a further 5 days to allow for possible irradiation damage repair mechanisms to manifest themselves.

Sterility tests (practical applications)

Irradiated cultures were tested by transferring 10 µl loopfuls to 4 bijou bottles each containing 2.5 ml of BHIB. Two of these were heated to 62.5 °C for 15 min. All four were incubated at 37°C for 7 days and then subcultured onto BA plates in a search for growth of *B. anthracis*.

Formaldehyde inactivation

Twenty millilitre volumes of Ames and Sterne strain spore suspensions at a concentration of $1x10^9$/ml were incubated in 5% formaldehyde at 37 °C for 24 h. The suspensions were centrifuged at 10,000 x g for 10 min and the pellets resuspended to their original volumes in SDW. To check for sterility, serial dilutions of the suspensions were plated on nutrient agar; 2 ml aliquots were then incubated in 500 ml of nutrient broth at 37 °C for 48h followed by subculture on nutrient agar.

Electron microscopy

Irradiation- or formaldehyde-killed spores were washed in 100 mM phosphate buffer, pH 7.4, and fixed overnight at 4 °C in 10% glutaraldehyde in 100 mM phosphate buffer. After a further wash in buffer, the spores were subjected to a further fix in 1% osmium tetroxide at 4 °C for 1-2 h. The suspensions were centrifuged and the pellets of spores embedded in 2% Difco agar. The agar pellets were dehydrated through graded methanol dilutions, soaked in neat propylene oxide for 3 h, and then for a further 3 h in a 50% mixture of propylene oxide and embedding resin (Spurr epoxy resin). The pellet was then cut into 1 mm cubes and embedded in embedding resin. After curing, sections were cut with an ultramicrotome and stained with uranyl acetate and lead citrate for examination.

Results

Inactivation of vegetative bacilli - systematic study

Suspensions of vegetative forms of the Sterne and Ames strains at a starting concentration of $1x10^7$/ml were sterilised by doses of 2.2 to 2.4 kGy (Fig 1). There was a 3% level of spore contamination but this did not appear to influence the dose needed to sterilise the suspension.

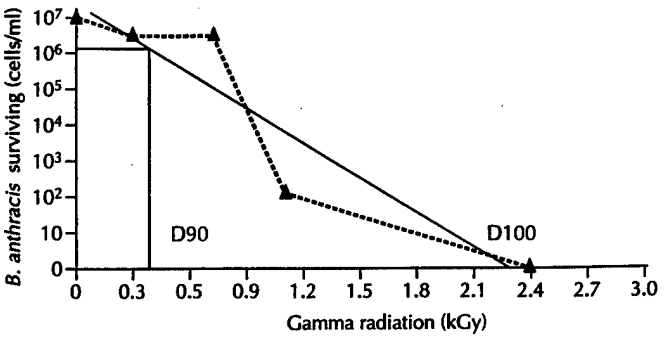

Figure 1. Inactivation of *B. anthracis* (Sterne) vegetative cells with gamma irradiation. The sterilising dose (D100) is predicted to be 2.3 kGy. The D90 was predicted to be 0.45 kGy. The curve was precisely similar in duplicated tests on both the Sterne and Ames strains.

For sterilization of foods, the Food Council recommends application of the dose calculated to produce 100% kill (D100) plus the dose needed for 90% kill (D90) to compensate for errors in the viable count procedure. In these tests, this amounted to 2.4 + 0.45 kGy. Applied to the vegetative cell Ames and Sterne strain preparations, this dose invariably resulted in sterility of the suspension.

Inactivation of spores - systematic study

The predicted D100 for a 10 ml suspension of spores at a concentration of $1x\ 10^9$ spores/ml was 36 kGy (Fig. 2). This is 15-fold higher than the D100 for vegetative forms, although these had been at a lower concentration at the time of exposure. The D90 was reached at a fairly early stage of treatment (5.5 kGy) and prolonged treatment was required to kill the remaining 10%. Agitation to eliminate any bulking effect, i.e., the surviving spores being protected from the irradiation as a result of physical shielding by other spores, did not alter the kill rate. For practical purposes, and in accordance with the Food Council guidelines, 41.5 kGy appeared to be the appropriate dose to apply in order to obtain the complete inactivation of spores of *B. anthracis*. The Mainance gamma source took 50 h to deliver this dose.

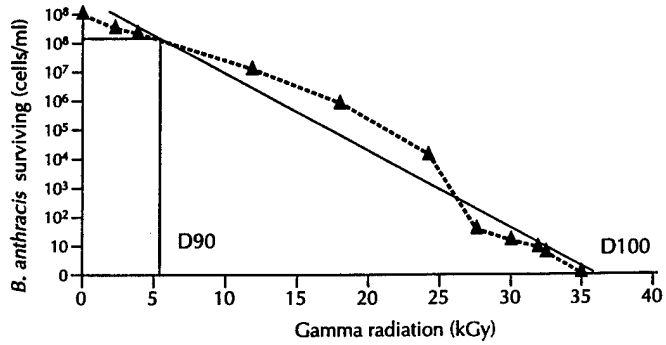

Figure 2. Inactivation of *B. anthracis* (Ames) spores with gamma irradiation. The sterilising dose (D100) is predicted to be 36 kGy. The D90 was predicted to be 5.5 kGy.

Electron microscopy

There were no indications that spores subjected to high doses of irradiation suffered any obvious physical damage. A comparison between irradiated and formaldehyde showed no obvious morphological differences (Fig. 3).

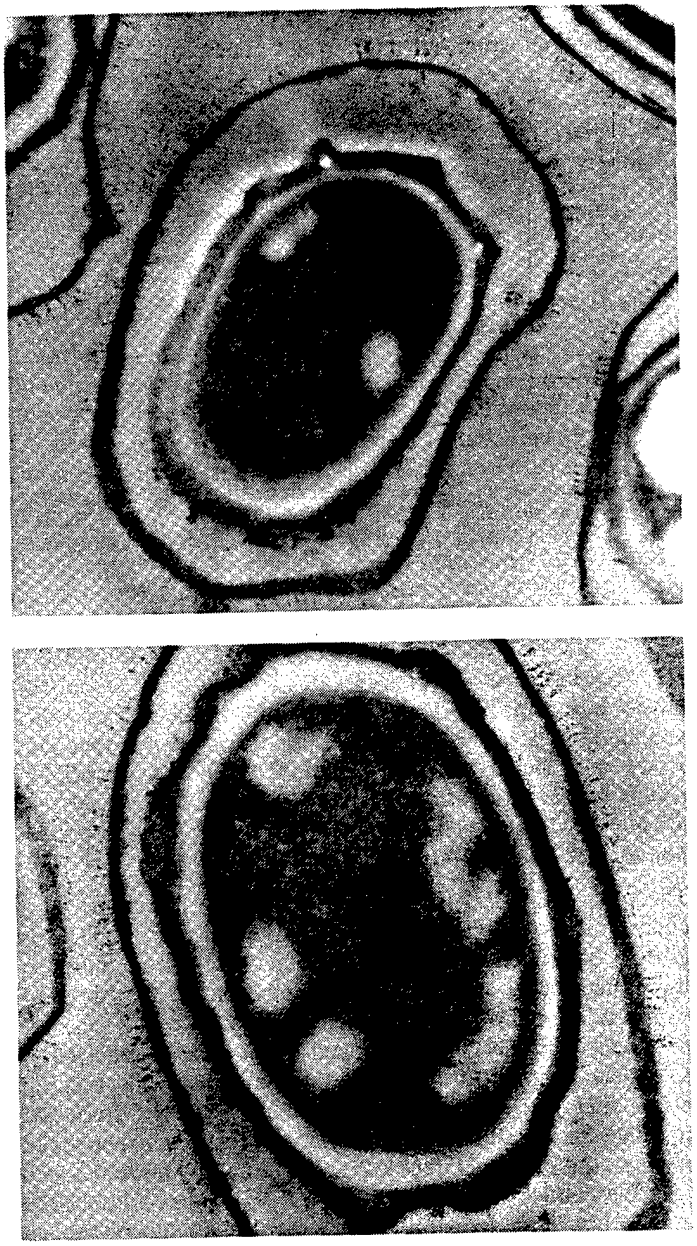

Figure 3. An electron microscope comparison of formalin-inactivated (top) and radiation-inactivated (bottom) spores showed no obvious morphological differences. (x 53,000)

Inactivation of vegetative bacilli and spores - practical application

Of the 38 strains used, totally spore free vegetative cell preparations were obtained for 16 (including the Ames strain). Spore levels in the remaining 22 ranged from just detectable with BHIB enrichment (the Sterne strain was among these) to 0.05% of the total viable counts which ranged from $1x10^8$ to $2.5x10^{10}$ cfu/ml. In an initial irradiation session, on the basis of the customer stated requirement, these suspensions were exposed to 10.6 kGy. Sixteen (not including the Ames and Sterne strains) failed the sterility tests; two of these had been recorded as spore-free vegetative preparations.

Based on the guidance provided by the results of the systematic study, the 16 vegetative cell preparations which failed the sterility tests and all the spore suspensions were exposed to 44 kGy. One of the vegetative cell preparations (ASC 181) and 7 of the spore preparations (ASC numbers 42, 79, 246, 267, 273 and 274 and Russian vaccine strain STI) failed the sterility tests. The failures were, however, "minor"; i.e., growth occurred in only 1 of the 4 BHIB cultures. Initial spore counts in the 39 suspensions ranged from $1.3x10^9$ to $3.4x10^{10}$. Sterility failure suspensions had had initial counts of $5.3x10^9$ to $1x10^{10}$.

Readings of 44.2 kGy by the dosimeter outside the SAF-T-CASE, 44.0 kGy by the dry internal dosimeter, and 44.3 kGy by the immersed internal dosimeter, indicated that the target doses had been effectively achieved. The Isotron source delivers doses at the rate of about 5 kGy/h.

Discussion

By and large, the guidance produced by the systematic study that a dose in the order of 41.5 kGy would be necessary to sterilize anthrax spores was effective in the subsequent practical application. This is in line with the reported sterilizing doses for other *Bacillus* species[1]. However, the minor sterility check failures on eight of the preparations indicated that even 44 kGy cannot be assumed to produce absolute sterility in an irradiated spore suspension. The killing effect of the irradiation was not critically affected by the volumes (2.5, 5-6, or 10 ml) of the spore or vegetative cell suspensions being irradiated.

A comparison of spore counts indicates that resistance to the killing effect of irradiation is strain related.

Repair and recovery by microorganisms following irradiation is well documented and the long incubation period (7 days) used in the sterility checks was to allow for this.

Examination of the spore structure after irradiation in the systematic study revealed no obvious differences in morphology as compared formaldehyde-killed spores. Previous experience has shown that heat- or formaldehyde-killed vegetative *B. anthracis* cells or spores are unsuitable for the production of specific antisera.

Data are not yet available in the performance of irradiated equivalents in this regard but client permission has been obtained to report that the results of preliminary tests on the irradiated cells and spore preparations from the 38 strains indicate that they react with various anthrax-specific polyclonal and monoclonal antibodies in a normal manner.

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Resistance to aldehyde disinfectants of *Bacillus* species spores

D. VIDAL, F. DESOR, D. ROVARCH and M. RICHARD

Centre de Recherches du Service de Santé des Armées, Unité de Microbiologie, 38702 La Tronche, France

Introduction

Sporulation is the dormancy state of a limited number of Gram positive bacteria of the genera *Bacillus* and *Clostridium*. Among *Bacillus*, some species are pathogens for man, such as *B. cereus* and *B. anthracis*. Sporulation is a differentiation mechanism which confers on these bacterial forms an extraordinary resistance to physical and chemical agents[2]. Spores can be inactivated by sterilisation processes or by using sporicidal disinfectants. The efficacy of sporicidal agents is

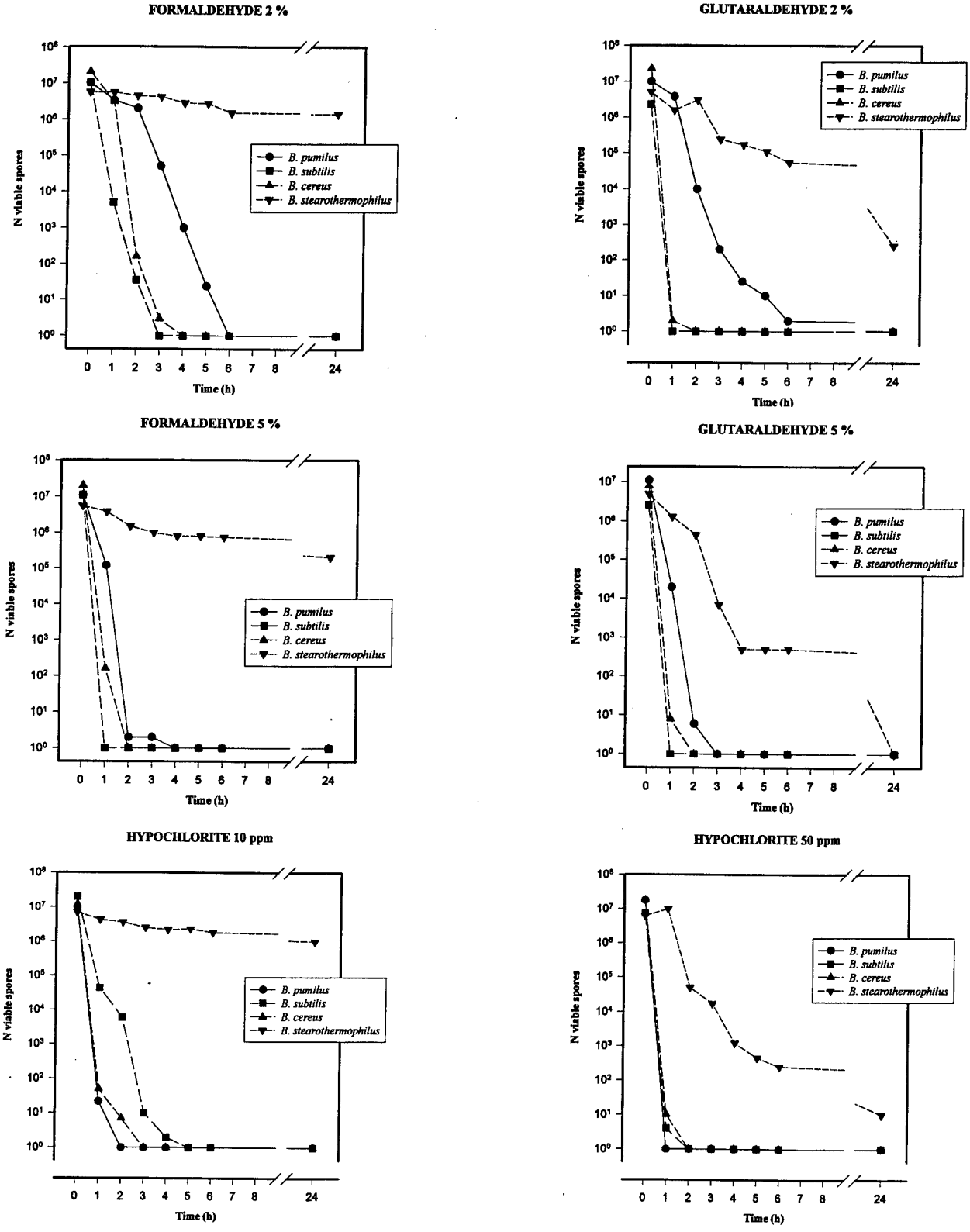

Figure 1. Time killing curves of *Bacillus* spores (10^7 spores/ml) exposed to glutaraldehyde (2 or 5 %) or formaldehyde (2 or 5 %) or sodium hypochlorite (10 or 50 ppm) at 20°C for a time of contact ranging from 30 minutes to 24 hours.

evaluated according to national standard methods based on contact between spore suspension (*Bacillus subtilis*, *B. cereus*, and *Clostridium sporogenes*) and disinfectant for a given period of time and at a given temperature. After elimination of the disinfectant, viable spores are enumerated on agar plates. This work was carried out to investigate the sensitivity of spores of *Bacillus subtilis*, *B. cereus*, *B. stearothermophilus* and *B. pumilus* to aldehyde disinfectants in comparison with hypochlorite.

Methods and results

Spore suspensions (10^7 spores/ml) were exposed to glutaraldehyde (2 or 5 %) or formaldehyde (2 or 5 %) or sodium hypochlorite (10 or 50 ppm) at 20°C for a time of contact ranging from 30 minutes to 24 hours. They were filtered through 0.2 µ membranes and washed before plating on culture medium. Controls were done for evaluating the spore titre without sporicide agent and for verification of elimination of the sporicide agent from the membrane. The 2% formaldehyde solution gave a 10^5 reduction in spore numbers in 2 hours with *B. subtilis* and *B. cereus*, and in 5 hours with *B. pumilus*. The 2% glutaraldehyde solution gave a 10^5 reduction of the spore number in 1 hour with *B. subtilis* and *B. cereus*, and in 3 hours with *B. pumilus*. With 10 ppm sodium hypochlorite the 10^5 reduction was obtained in 1 hour for *B. pumilus* and *B. cereus*, and in 3 hours for *B. subtilis*. In all these conditions *B. stearothermophilus* was highly resistant. The 10^5 reduction was obtained with 50 ppm sodium hypochlorite in 6 hours (Figure 1). By transmission electronic microscopy, *B. pumilus* spores are very small in comparison with *B. subtilis* and *B. cereus* spores. *B. pumilus* spores show a characteristic aspect with a multi-layered dense coat but without exosporium. In contrast, *B. cereus* spores are surrounded by an exosporium with a less dense coat. These morphology differences may explain the different resistance to aldehyde disinfectants (results not shown).

Discussion and conclusions

The relative resistance of spores to disinfectants is a complex phenomenon in which the spore coat plays an important role in limiting the disinfectant penetration to the underlying cortex and protoplasm. Some agents, such as hypochlorites, aldehydes and peroxides, are able to degrade these outer layers and are efficient in destroying the spores though they are even more active than on vegetative forms. Our results show that glutaraldehyde is more effective as a sporicidal agent than formaldehyde.

B. stearothermophilus is extremely resistant to aldehydes and hypochlorite. This micro-organism is known to produce spores highly resistant to physical agents, such as radiation and heat. Our results reveal its high resistance to chemical agents in comparison with other *Bacillus* spores. *B. pumilus* is more resistant to aldehyde than *B. subtilis* and *B. cereus*. In contrast, *B. pumilus* is more susceptible to hypochlorite. *B. pumilus*, sometimes the cause of infection in the immunocompromised patient[1], should be considered as a biological indicator for testing the efficacy of sporicidal aldehyde disinfectants. This study will be extended to spores of *B. anthracis*, *Clostridium* and *Aspergillus* species.

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Disinfection against spores of *Bacillus anthracis*

M.N. JONES and P.C.B. TURNBULL

Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG

Summary

As part of an attempt to address new regulatory requirements that evidence of the effectiveness of decontamination procedures in production facilities be demonstrated with the specific organisms in use, the sporicidal efficacies of a number of disinfectant formulations against spores of the Sterne vaccine strain of *Bacillus anthracis* and against spores of the generally used simulant, *B. subtilis var. globigii* (NCTC 10073), were compared using the Kelsey-Sykes capacity test.

The formulations meeting the Kelsey-Sykes criteria of a 5-log fall in viable counts within 1 h in the case of the Sterne strain spores were 10% Chloros™ in water acidified with 8 ml/litre concentrated HCl, 25% Chloros in absolute alcohol, 75% Chloros in 50:50 alcohol:water, 75% Chloros in alcohol acidified with 8 ml/litre concentrated HCl, and 8% formaldehyde in absolute isopropanol. The most rapidly cidal was 75% Chloros in acidified alcohol. (Methanol and ethanol appeared to be interchangeable in these formulations.) In addition, 10% aqueous formaldehyde solution, 2% and 5% glutaraldehyde in 0.3% $NaHCO_3$, and 3% and 5% peracetic acid reduced starting spore levels of 8.5×10^7 to 7.7×10^8 cfu/ml to undetectable within 2 to 3 hours. Commercial formaldehyde- and glutaraldehyde-based disinfectant Tegodor™, and Virkon™, a peroxygen-based formulation, were ineffective at the concentrations recommended by the manufacturers while hydrogen peroxide was only slowly effective when used as a saturated solution.

In comparison with the *B. anthracis* spores, the *B. globigii* spores appeared significantly more susceptible to lower concentrations of Chloros™, formaldehyde and peracetic acid solutions and somewhat more susceptible to Tegodor™, Virkon™ and hydrogen peroxide. The results suggest that the value of *B. globigii* spores as simulants in efficacy tests on disinfection procedures for the Sterne strain of *B. anthracis* is limited.

Introduction.

Disinfection and decontamination procedures in use in production areas and laboratories have frequently been designed and assessed in the past on the basis of their effectiveness with simulant organisms. In the case of *Bacillus anthracis* and other sporulating bacteria, efficacy tests for disinfection and decontamination procedures have traditionally utilised *B. subtilis* var *globigii* (NCTC 10073). Regulatory requirements now demand evidence of the effectiveness of decontamination procedures for the specific organisms used within such facilities.

In the two production units at CAMR, used for the manufacture of a variety of licensed and unlicensed material, decontamination and disinfection is carried out between consecutive production processes to prevent the cross-contamination of products. The production of the UK human anthrax vaccine utilises the Sterne vaccine strain ($34F_2$) of *B. anthracis* (NCTC 8234).

This paper presents the results of a preliminary study to establish the extent to which decontamination procedures, hitherto validated using spores of *B. globigii*, were effective against spores of *B. anthracis* var. *Sterne*.

Materials and methods

Disinfection validation test method

The method used was the modified Kelsey-Sykes test[11] (BS 6905:1987). This is a capacity test developed to measure the ability of a disinfectant to retain activity under a wide range of conditions, including the presence of proteinaceous material. The arguments in favour of choosing this disinfectant efficacy test are given elsewhere[2].

Ten millilitre volumes of freshly formulated disinfectant were added to equal volumes of a Sterne strain spore suspension to give initial counts of 8.5×10^7 to 7.7×10^8 cfu/ml. The spores were prepared as described elsewhere in this volume[9]. At 5, 15, 30 and 60 min after exposure to the disinfectant, 1 ml aliquots were removed and added to five 9 ml volumes of appropriate neutralising solution. In a portion of the tests, aliquots were taken at 120 and 180 min also. After a 10 min exposure to the appropriate neutraliser, one of the five reaction tubes was used to carry out serial dilutions in 0.1% peptone and duplicate 100 µl aliquots of each dilution were plated on 7% blood agar (BA) followed by incubation overnight at 37°C. The remaining four neutralisation tubes were incubated overnight at 37°C before being subcultured on BA to look for survival of the test organism where not detected on the count plates.

The disinfectants tested and their respective neutralisers are listed in Table 1.

Table 1. Disinfectants tested and the respective neutralisers used

Disinfectant	Neutralisation Buffer
Chloros™	1.8% sodium thiosulphate
Tegodor™	0.1% histidine
Virkon™	1.8% sodium thiosulphate
Hydrogen peroxide	catalase in distilled water
Glutaraldehyde	1% glycine
Formaldehyde	0.1% histidine
Peracetic acid	0.2% sodium thiosulphate

Results and discussion

The modified Kelsey-Sykes test specifies that a successful disinfectant should achieve a 5-log reduction in viable counts within one hour. The majority of the tests were performed in triplicate.

The results obtained with the different disinfectants were as follows.

Chloros™

The sporicidal activity of hypochlorite solutions has been shown to be dependent on the availability of free chlorine,[4,6] and the pH of the solution with free chlorine is reported to be more effective against spores under acidic conditions[4,6,14,18]. The sporicidal activity of hypochlorite solutions, including Chloros, has also been reported to be enhanced in alcoholic solutions[10].

The diluents for Chloros (Robert R. Bartlett & Sons Ltd, Yate, Bristol, UK) used in the present series of tests were deionised water, phosphate citrate buffer (pH 4), 50:50 methanol or ethanol:deionised water, absolute methanol or ethanol, deionised water acidified by addition of 8 ml/litre of concentrated hydrochloric acid (with resulting pH of about 1.5), and methanol or ethanol also acidified by addition of 8 ml/litre of concentrated hydrochloric acid.

Complete kill of the *B. anthracis* spores within one hour did not result from exposure to Chloros in any of the diluents, but a 5-log reduction was achieved with 10% Chloros in the acidified water, 25% in absolute alcohol, 75% in 50:50 alcohol:water and 75% in the acidified alcohol. The largest reduction in viable counts (7.6 logs) occurred in 75% Chloros in acidified water, but the most reproducible decrease (7 logs) was obtained with 75% Chloros in 50:50 alcohol:water. Methanol and ethanol were shown to be interchangeable in these diluents with no resulting differences in sporicidal activity.

In contrast to the results with the *B. anthracis*, a 7.8-log reduction in viable *B. globigii* to undetectable resulted within 30 min upon exposure of the spores to 10% chloros in all of the diluents.

A comparison between the results obtained with the routinely used 10% Chloros in water and the formulation regarded as the most effective, 75% in 50:50 alcohol:water, is shown in Fig. 1.

The stabilities of the formulations showing optimal sporicidal activities were not tested; this would have to be done before they could be recommended for use.

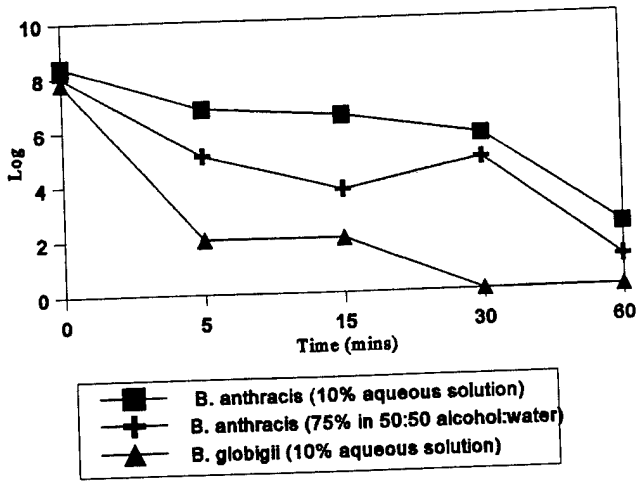

Figure 1. Chloros

Formaldehyde

Formaldehyde in aqueous solutions is the sporicide routinely used in CAMR, though normally for the purposes of fumigation by boiling off 10% formalin (4% formaldehyde) solutions[12]. For disinfection of surfaces or materials, 5% formaldehyde solutions proved effective against anthrax spores[13], but >2 h was required for fully effective kill[5,16]. The use of isopropanol has been recommended as the diluent with 8% formaldehyde in isopropanol being reported as more sporicidal than a 10% solution in water[7]. The results of tests comparing these formulations are presented in Figs 2, 3 , and show that a 4.5-log fall in *B. anthracis* counts was achieved in 60 min with 10% formaldehyde (1/3 v/v undiluted formalin/sterile deionised water) in water (Fig. 2), and a 5.5-log reduction with 8% formaldehyde in isopropanol (Fig. 3). The latter therefore achieved the Kelsey-Sykes target for a successful disinfectant against the Sterne strain spores.

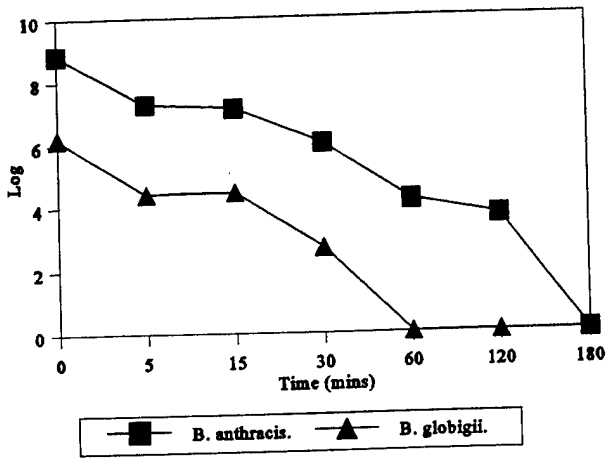

Figure 2. Formaldehyde (10% in water)

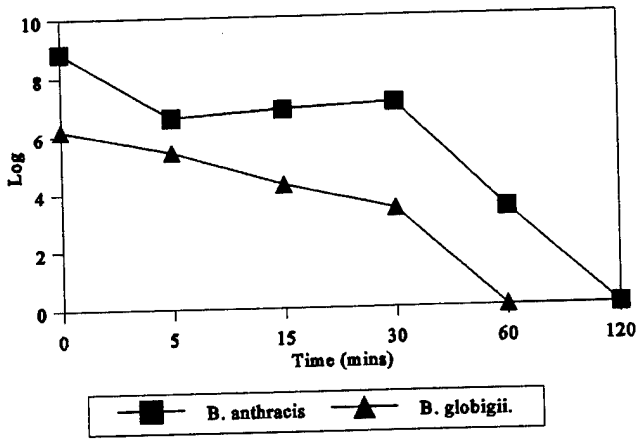

Figure 3. Formaldeyhde (8% in isopropanol)

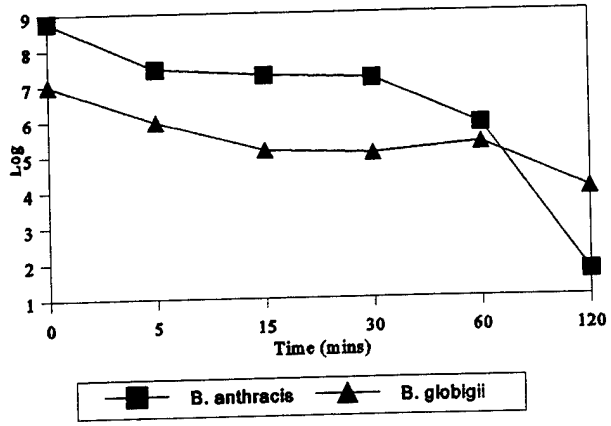

Figure 4. Glutaraldehyde (2%)

With both formulations, the *B. globigii* spores were reduced to undetectable in the 1 h period and the *B. anthracis* spores to undetectable by 2 h.

Glutaraldehyde

As with Chloros^T, the pH of the buffer system used is critical to the sporicidal activity of the glutaraldehyde; with glutaraldehyde, however, the pH has to be alkaline rather than acidic[15,16,17]. Sodium bicarbonate is thus an appropriate buffer system and glutaraldehye and sodium bicarbonate (0.3% w/v) are the principal ingredients in the commercial disinfectant, Cidex^T ("activated gluatarldehyde").

Concentrations of 1% and 2% glutaraldehyde at pH 8 were shown by Rubbo *et al.*[16] to be markedly superior to 4% aqueous formaldehyde against spores of *B.anthracis*, reducing a suspension of 10^9 cfu/ml to undetectable within 15 and 30 min respectively.

The results from the present tests using 2% and 5% glutaraldehyde in 0.3% NaHCO3 are shown in Figs. 4, 5. Declines in viable *B. anthracis* spores following 1 h exposures to these concentrations failed to achieve the kill rate reported by Rubbo *et al.*[16]. The contrasting results are probably attributable to the manner in which the spores were prepared, and possibly, to a lesser extent, to strain differences. The spores of Rubbo *et al.* were simply prepared by harvesting 5-day cultures of an avirulent strain on nutrient agar; a much less stringent procedure than that used in CAMR.

In the present study, the 5% glutaraldehyde solution almost achieved the Kelsey-Sykes criteria of an effective disinfectant but the full 5-log decrease in viable counts of *B. anthracis* was only obtained after exposure for 2 h (Fig. 5).

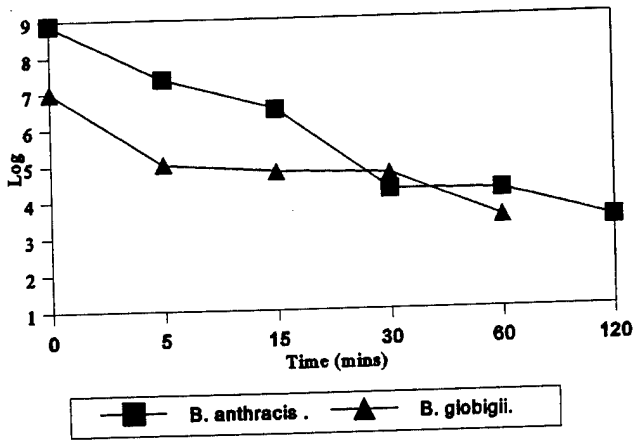

Figure 5. Glutaraldehyde (5%)

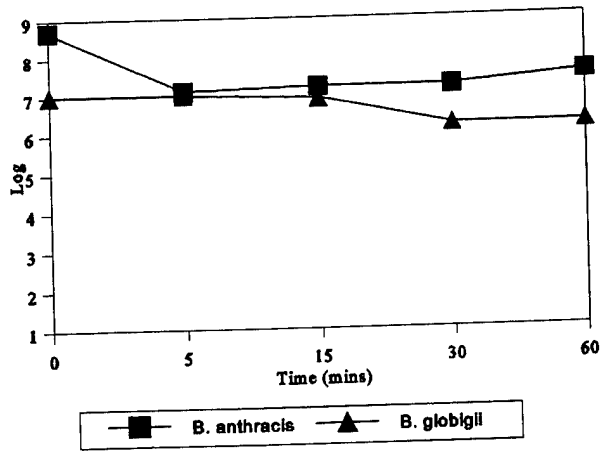

Figure 6. Hydrogen peroxide (4%)

Hydrogen peroxide

Oxidising agent hydrogen peroxide is well established as bactericide and sporicide[1,3,5]. Three or four per cent solution have been shown to be effective against anthrax spores und some conditions[3,4].

Results obtained in this series of tests with 4% and 30 (saturated) solutions of hydrogen peroxide in water are show

in Figs 6, 7. Exposure to 4% hydrogen peroxide for 1 h resulted only in a 1.2-log reduction in the viable count of the *B. anthracis* spores while a 4-log decrease resulted from exposure to the 30% solution. Similar results were obtained with the *B. globigii* spores. A 1-log reduction resulted from exposure for 1 h to the 4% solution and a 4-log decrease from exposure for 1 h to the 30% solution.

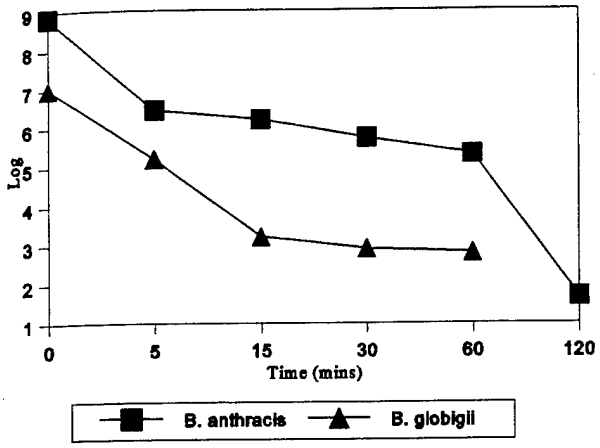

Figure7. Hydrogen peroxide (30%)

Peracetic acid

Peracetic acid in a 3% solution has been shown to be sporicidal against anthrax spores[3,8,13]. The results presented in Figs. 8, 9 show that, while 3% peracetic acid reduced the *B. globigii* spores to undetectable in just 5 min, it reduced the *B. anthracis* spore counts by just 2.5 logs in 1 h (Fig. 8). A 5% solution reduced the *B. anthracis* spore count by 3.2 logs (Fig. 9)

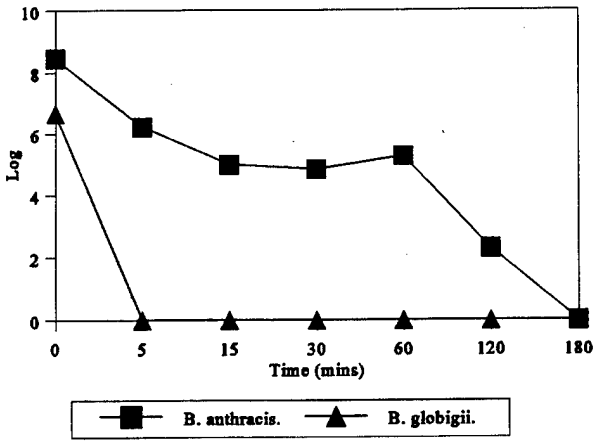

Figure 8. Peracetic acid (3%)

Tegodor™

Tegodor (Th Goldschmidt Ltd, Ruislip, Middx, UK) is a commercial mixture of formaldehyde, glutaraldehyde and quaternary ammonium compounds in aqueous solution. The manufacture recommends use at 0.5-1% in deionsed water for surface disinfection and at 3% when used as an aerosol. Sporicidal activity on both *B. anthracis* and *B. globigii* spores were negligible when used at these concentrations (Fig. 10). Other concentrations examined were 5%, 10%, 50%, and undiluted with exposure for 1 h. The greatest effect on the Sterne strain spores was a 3.3-log reduction in counts obtained with 50% Tegodor. This contrasts somewhat with the results with formaldehyde and glutaraldehyde by themselves (above), especially the former. The concentrations of these agents in Tegodor and the possible counteractive effects of the three ingredients on each other are not known.

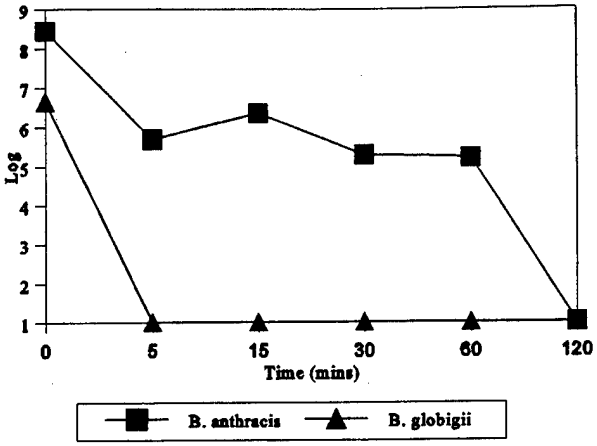

Figure 9. Peracetic acid (5%)

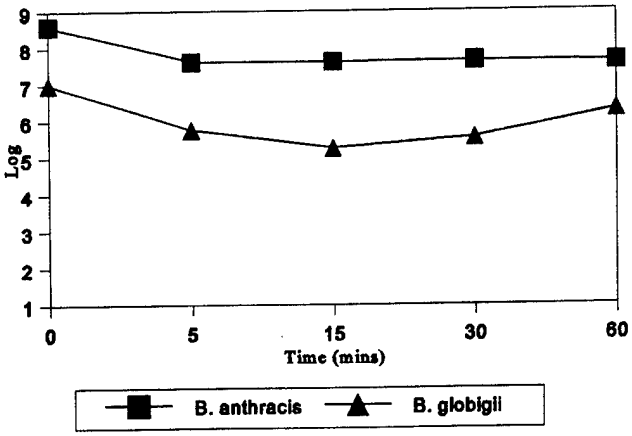

Figure 10. Tegodor (1%)

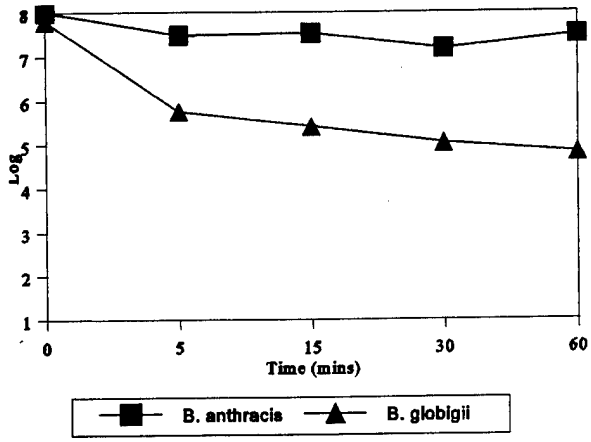

Figure 11.Virkon (1%)

Virkon™

Virkon, another commercial disinfectant (Antec International Ltd, Sudbury, Suffolk, UK), consists of "peroxygen compounds, inorganic salts and surfactants", was included as a disinfectant in

current use at CAMR. Tested at its recommended dilution of 1%, essentially no sporicidal effect was detected on the *B. anthracis* spore preparation (a 0.5-log reduction over 1 h) while there was approximately a 3-log fall in viable counts on the similarly exposed *B. globigii* spores (Fig. 11).

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Disinfection and decontamination of animal fibres: connected problems and state of the art

*C. BRINI[1], F. PIUNTI[1], R. TERZI[1], C. TONIN[2], R. INNOCENTI[2], A. MOIRAGHI[3] and S. DI TOMMASO[3]

*Servizio Veterinario, via P. Maffei 59 13014 Cossato BI Italy. [2]C.N.R. Istituto di Ricerche e Sperimentazione Laniera "O. Rivetti" Biella, Italy. [3]Università degli Studi di Torino, Istituto di Igiene *(authors unable to attend the workshop)*

The processing of luxury animals fibres, like cashmere, mohair, camel and camelid hair, represents a resource of main importance for Biella (northern Italy) and its surrounding area.

Wool workers who handle raw fibres are constantly exposed to infectious diseases, which potentially may affect the skin, lungs or intestines. In 1987 and 1988 two outbreaks of industrial anthrax (wool sorter's disease) took place, affecting two textile workers employed in a cashmere dehairing and carding mill near Biella, with one fatal outcome.

Workers engaged in the early stages of fine animal fibre processing are exposed to anthrax infection more continually than other wool workers, since the raw fibre lots are imported from Asiatic countries where the disease is prevalent. Considering that the requirements offered by international health certificates often do not provide an absolute guarantee of safety for the raw animal fibres, (i.e. that the raw wool or hair is free from bacillary and/or spore forms of *Bacillus anthracis*), all lots imported from known or suspected dangerous areas must be disinfected compulsorily, under sanitary supervision. The possibility of disinfecting and/or decontaminating raw animal fibres near the Italian mills and of establishing a disinfecting station in Biella's industrial district, is of great interest to both businesses and the Prevention Department (a Department of Italian National Health Service, composed of three Services: Public Health, Veterinary Public Health, Industrial Hygiene).

World-wide there is no generally agreed legislation concerning the control of animal materials that may be potentially infected with *Bacillus anthracis*. Also the International Zoosanitary Code of the International Office of Epizootics of Paris require that: "On the application of the measures provided for in this Code, veterinary administrations of importing countries should require the presentation of an International Sanitary Certificate attesting that "...the products have been subjected to treatment likely to destroy both bacillary and spore forms of *Bacillus anthracis*". This statement implies that it is of utmost importance to find out the best disinfection and/or decontamination techniques and the methods for testing the efficiency of the sterilization process. However, in Europe, there is only one functional disinfection station for wool and hair utilising formaldehyde treatment. With environmental protection problems posed by formaldehyde in mind, some tests have been made on different disinfecting agents, such as ethylene oxide (EtO), steam, gamma irradiation.

Particular stress should be placed on the relevant economic value and on the intrinsic delicacy of the luxury animal fibres, like cashmere: the disinfection and/or decontamination methods must not damage the fibres, nor leave residues of any kind in the products.

A product is sterile when no living organisms are present. To check the result of the sterilization, the product can be seeded with spores of *Bacillus subtilis* var. *niger* (ATCC 9372) and tested to determine their survival.

Ethylene oxide sterilization

The product is placed in a sealed container in which it undergoes a cycle of temperature, humidity and pressure that ensures its complete penetration, even if already packaged, by the sterilization gas (EtO).

The sterilization process parameters are an initial vacuum level 0.30 bar; holding time under vacuum 30 min; steam soak time 10 min; EtO injection - pressure rise 2.8 bar, time to achieve it 30 min, holding time (minimum) 20 min; gas concentration in chamber 370-410 mg/l; chamber temperature 40°C; relative humidity 50% ±10%; sterilization time 180 min; degassing - number of vacuum pulses 8, pressure 0.3 bar.

Gamma ray sterilization

The product is placed close to a source of radioactivity (normally cobalt 60), so as to be irradiated until the total amount of radiation absorbed reaches a preset level that is high enough to reach the product sterility (normally 2.5 Mrad). Typical duration of irradiation: from 2 to 24 hours.

Table1. Results of preliminary trials on method for sterilizing delicate and valuable raw and scoured wools.

Wool	Gamma Ray	Steam	EtO
Test 1			
Wool top, Australia	+	-	-
Wool top ex USSR	+	+	-
Raw Wool Australia	+	-	-
Cashmere Iran	-	+	-
Cashmere China	+	+	-
Cashmere Mongolia	+	-	-
Cashmere Afghanistan	-	+	-
Test 2.			
Wool, Aus 19.2 -m (fleece)	Steam	EtO	
Greasy	+	-	
Scoured	-	-	
Carded silver	-	-	
Tops	-	-	
Tops (DCM)	-	-	
Tops (DCM + ET)	-	-	

(+, not sterilized; -, sterilized)

Steam sterilization

The product is placed in an autoclave, where it undergoes a heat cycle. Normally steam at 110-120°C is used for approximately forty to fifty minutes.

A preliminary study was done to define an affordable method for the sterilization of delicate and very expensive animal fibres like cashmere, and also to investigate the feasibility of constructing a sterilization station for all raw animal fibres, including raw wool.

In the course of the study various samples of animal fibre, were tested including wool, raw and scoured, from Australia the former USSR, and cashmere, from Iran, China, Mongolia, and Afghanistan. The differences between the results obtained with different techniques are shown in Table 1.

These differences may be attributed partly to the characteristics of the test conditions, for example, different steam temperature (115-120°C) and probably to the intrinsic quality of the raw fibres, which are extremely rich in fat and high moisture content. However, since the origin, cleanliness and physical characteristics of the lots may have an influence on the conditions of the test, it must be considered that these results would vary from sample to sample.

Further studies must be done, investigating if the delicate fibres are damaged by the sterilization process, or if some residue of the ethylene dioxide and some of its derivatives will remain in the products, producing technological and health problems.

Bacteriology, serology and pathology of experimental anthrax in pigs

C. REDMOND, G.A. HALL, M. GREEN and P.C.B. TURNBULL

Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, UK

Summary

In order to study anthrax carriers, their possible risk to public health, and disease pathogenesis, 50 pigs were challenged perorally with *Bacillus anthracis* spores inoculated into their feed. Two of the pigs died of anthrax 6 and 8 days respectively after challenge but the remainder, observed over a 21-day period following challenge, only exhibited mild and transient clinical signs of disease. As judged by the results of bacteriological culture of appropriate tissues from the survivors, it was concluded that meat from healthy pigs killed 21 days after the latest case during an outbreak does not pose a public health risk.

Introduction

An outbreak of anthrax in a 500-sow herd in the UK in 1989 caused 19 deaths due to the disease and 4,492 pigs were slaughtered and incinerated as contacts[4, 21]. The outbreak lasted 95 days, possibly due to the presence of carriers. The pathogenesis of anthrax in pigs is poorly understood and the existence of carriers and the risk they pose to public health have not been investigated fully. There is, however, no evidence that consumers in the UK have ever been infected from handling or eating porcine products, even in the days when anthrax in pigs was much more common than it is now. Very few records of experimental anthrax infection in pigs exist and those that were found[8, 11, 19, 20] supplied incomplete information on these aspects.

The aim of the study therefore was to establish if infection persisted in the pigs that had recovered from clinical disease and to evaluate the risks to public health of moving pigs from a unit for immediate slaughter. This paper describes aspects of the bacteriology, pathology and pathogenesis of the experimental disease.

Materials and methods

Strain and dose

Spores of the Ames strain of *B. anthracis* prepared as described on page 126 were used throughout the study in the absence of an isolate from an episode of anthrax in pigs. The Ames strain was chosen because of its established virulence for experimental purposes in guinea pigs[9,17]. In the case of the three control pigs, the spores were killed by irradiation. The initial dose was 10^7 cfu progressing to 10^{10} in successive infection sessions. Passaged isolates from the two pigs that died used for 3 subsequent infection sessions, did not possess obviously increased virulence.

Challenge

Ten groups (1-10) of 12-week-old cross-bred pigs, 5 per group, were challenged orally. The aim at the outset was to induce lethal clinical disease in one animal in each group, leaving 4 to be monitored for 21 days, before comprehensive examination at necropsy. Three pigs were challenged with the irradiated spores to serve as controls. Grit was included in the diet to facilitate infection 5 days before and for 2 to 3 days after challenge.

Specimen collection

Rectal temperatures were measured daily and the pigs monitored for clinical signs of illness.

Faecal and saliva samples were collected for culture on days 1, 2, 4, 8, 11, 16 and 21 following infection. Blood samples were collected for culture and serology before each challenge and at necropsy on day 21 in all pigs and also on days 2, 4 and 7 in a proportion of animals. Also at necropsy, samples of peritoneal fluid, submandibular, prefemoral, prescapular and mesenteric lymph nodes, liver, kidney, spleen and lung were taken for culture and histopathology. Tissues for histopathology were fixed in formalin and processed to sections stained with haematoxylin and eosin.

Culture

Culture consisted of standard procedures for isolation and confirmation of *B. anthracis* as published[16]. Approximately 30 g of faeces were suspended in 2 volumes of sterile deionised water (SDW). Volumes (100 µl) of 10-fold dilutions down to 10^{-3} were spread on 7% horse blood agar (BA) and polymyxin-lysozyme-EDTA-thallous acetate (PLET) plates before and after heating at 62.5 °C for 15 min. In the case of the pre-heated samples, the BA contained 7 mg/ml polymyxin (BAP) to reduce growth of miscellaneous non-sporing bacteria. *B. anthracis* was looked for on the BA and BAP plates after overnight incubation at 37 °C and on the PLET plates after approximately 36h at 37 °C.

Saliva swabs were agitated in 1-2 ml of SDW and then streaked over BAP and PLET plates. Alternatively, 100 µl aliquots of the agitate were spread over BAP and PLET plates before and after heating at 62.5 °C for 15 min followed by incubation and examination as above.

Blood samples were cultured by spreading a loopful of blood on a BA plate; a second loop was transferred to 10 ml of brain-heart infusion broth supplemented with 7 mg/ml polymyxin (BHIBP) for enrichment. Following overnight incubation at 37 °C, the enriched broth was subcultured onto a BA plate. All plates were incubated overnight at 37 °C.

Necropsy samples were chopped up using sterile scalpels and plated directly onto BA plates which were incubated overnight at 37 °C. If negative, approximately 1-2 g of macerated tissue was transferred to 10 ml BHIBP which, after overnight incubation at 37 °C, was subcultured on BA and PLET plates with incubation as before. In the case of the two pigs (1 and 25) which died of anthrax, in addition to culture of the blood and tissues, polychrome methylene blue (M'Fadyean) stained blood smears and impression smears of each tissue were viewed for the presence of encapsulated *B. anthracis*. Viable *B. anthracis* counts were obtained for the blood and mesenteric fluid samples.

Serology and toxin tests

The competitive inhibition enzyme immunosorbent assay (EIA) as previously described[17, 18] was used to determine the titre of the serum antibodies (all Igs) to the anthrax-specific Protective Antigen (PA) in all sera.

PA, as representative of the anthrax toxin, was looked for by capture EIA for antigen[15] in the sera collected at 2, 4 and, where taken, 7 days after challenge, this being the period when signs of illness were at their greatest.

Results

Clinical signs

Clinical signs of disease were non-specific or indicated gastrointestinal disturbance. Anorexia, lethargy, dullness, shivering, constipation, loose stools, blood in faeces and ataxia were detected in most animals. Two animals (pigs 1 and 25) died at 6 and 8 days respectively after challenge. Pig 1 had received an estimated 1.6×10^7 spores and pig 25 an estimated 7.8×10^7 spores derived from the pig 1 re-isolate. Thus they had not been exposed to the largest of the challenges. A lethal dose therefore was not defined.

Mean rectal temperatures were raised on days 2-5 post challenge and after returning to base level, they gradually increased from day 8 onwards (Fig. 1). Rectal temperatures in excess of 40 °C were taken as indicating pyrexia which was detected in 45 pigs, mostly on days 2-5 post challenge.

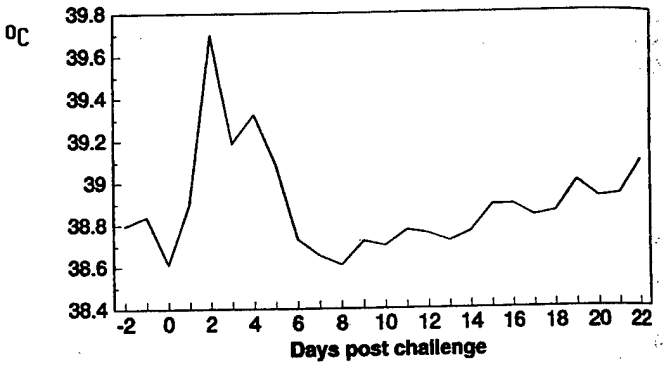

Figure 1. Mean rectal temperature readings

Culture, serology and toxin detection

In faeces and saliva, the numbers of *B. anthracis* isolated and the duration of isolation rose in parallel with increasing challenge doses. Following doses of 10^7 spores, faecal isolations were spasmodic by day 4, and no samples were positive on day 8. Following doses of 10^8, 10^9 and 10^{10} spores, spasmodic isolations were obtained 8-16 days post challenge. No saliva isolations were made prior to infection session 7, after which samples became consistently positive on days 1-4, with spasmodic isolations on days 8-16. Comparisons of viable counts before and after heating indicated that neither germination nor multiplication occurred in the mouth or gastrointestinal tract, in other words, that all these isolations represented ungerminated spores.

In blood, apart from pigs 1 and 25 which succumbed to infection, *B. anthracis* was isolated from only two blood samples (pig 43 at 2 days and pig 45 at 4 days), in both cases only on enrichment. Antibodies (all Igs) to Protective Antigen (PA) were not detected by competitive inhibition EIA in any of the 0, 2, 4 and 7 day sera. Marked seroconversion was apparent in the 21-day sera of the majority of the animals but 6 pigs (4 from infection session 4) were negative at 21 days and low titres were obtained in a further 6 pigs (3 from infection session 5). The anti-PA titres were, therefore, not obviously related to challenge dose.

PA was not detected by capture EIA in any of the 2, 4 and 7 day sera representing the period over which outward clinical signs of infection were observed.

At necropsy on day 21, just one specimen (lung of pig 47) yielded *B. anthracis* on direct plate culture (one colony on 11 BA plates) and 4 further specimens (liver of pig 19, liver and prescapular lymph node of pig 20, lung of pig 35 and the mesenteric lymph node of pig 45) were found to be positive by enrichment culture.

In the two pigs that died, *B. anthracis* was isolated from the spleen, liver, lung, kidney, mesenteric lymph nodes and prescapular lymph node, but not the submandibular or prefemoral lymph nodes. This is obviously significant to diagnosis of anthrax in pigs. The isolation results following the postmortems of the two pigs which succumbed to infection are presented in Table 1.

Pathology

Gross and microscopic pathology of pigs killed 21 days after challenge, in which *B. anthracis* was not detected, revealed hyperplasia of the germinal centres of the submandibular and mesenteric lymph nodes and small abscesses in 5/23 submandibular nodes and 5/33 mesenteric nodes. In one pig, intestinal adhesion to an adjacent mesenteric node was noted, intestinal adhesion to the abdominal wall was seen in another and jejunal perforation and abscessation was detected in a third. Infection in the alimentary tract did not appear to have spread beyond the draining lymph nodes, except in the pigs that died. Pathology in the pigs that died and in which infection was widespread indicated intestinal anthrax. Lesions consisted of fibrinous peritonitis, oedema of the colonic mesentery, necrosis and acute inflammation of the duodenum, acute necrotising lymphangitis and lymphadenitis of the pancreatico- duodenal lymph nodes.

Discussion

Most of the available data on anthrax in pigs dates from the early 1950's when, in both the UK and the USA, there seem to have been an unusually large number of anthrax outbreaks in swine[1, 2, 6, 7, 12, 13, 14]. It appears to have been accepted at this time that there were two principal manifestations of porcine anthrax; the pharyngeal and the intestinal forms. These were thought to reflect husbandry habits, the pharyngeal form being associated with the feeding of any food waste which inadvertently included meat and bones from one or more anthrax carcasses, and the intestinal form resulting from consumption of mineralised feed meals contaminated with anthrax spores.

The pharyngeal form was characterized by an ulcerative stomatitis, laryngitis and markedly oedematous swelling of the throat region, sometimes so extensive as to interfere mechanically with respiration, feeding and drinking. The infection was limited to the lymph glands of the pharyngeal and cervical regions[3, 5]. This form of porcine anthrax was recognized at the turn of the century[10].

Clinical signs of the intestinal form are regarded as less obvious than the pharyngeal form and, since it was recognized that recovery frequently occurred, it was thought that it often went unrecognized[1, 5, 6]. The symptom complex of the intestinal manifestation was reported to be digestive disturbance with anorexia, vomiting, diarrhoea (sometimes bloody) or constipation.

It would appear that the infection that occurred in the present experimental study exclusively took the intestinal form with non-specific clinical signs and gastrointestinal disturbance. Cervical oedema was never observed, and, in the two pigs that died, *B. anthracis* was not isolated from the submandibular lymph nodes. The histopathological findings were also commensurate with this conclusion.

The clinical observations and results of the serology indicate that transient infection occurred in most pigs. The isolations from the 5 pigs on day 21 after challenge needs to be viewed in the context of the actual numbers of *B. anthracis* involved. While extrapolation of the five positives on a statistical basis suggests that up to 10 % of the pigs might be expected to be infected 21 days after exposure in a herd experiencing an episode of anthrax, it seems more realistic to view the very low numbers of *B. anthracis* found as representing the final stages of clearance of the bacterium and the ability of pigs to overcome an enormous challenge.

The information from the 2 pigs which died indicates an incubation period of 6-8 days. Thus 21-day examinations at the end of each infection session can be thought of as having taken place 13-15 days after the last case. It was considered, therefore, that no significant risk existed from moving clinically healthy pigs for slaughter 21 days after the last case on an affected farm.

Acknowledgements

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Table 1. Postmortem in the pigs that died

Pig	Method	Blood	Peritoneal fluid	Spleen	Liver	Lung	Kidney	MLN	SMLN	PFLN	PSLN
1	Microscopy	++	+++	++++	+	-	N.E.	++	-	N.E.	N.E.
1	Culture (cfu/ml)	9.6×10^3	4.4×10^4	70%	++	+	N.E.	++	-	N.E.	N.E.
25	Microscopy	-	+	+	+	+	+	-	-	-	-
25	Culture (cfu/ml)	30	5.7×10^5	+++	+++	+	+	+	-	-	+

MLN, mesenteric lymph node; SMLN, submandibular lymph node; PFLN, prefemoral lymph node; PSLN, prescapular lymph node; N.E., tissues not examined

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Species differences in the pathology of wildlife in the Kruger National Park, South Africa

N. P. J. KRIEK[1] and V. DE VOS[2]

[1]Department of Pathology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, RSA, and [2]National Parks Board, Private Bag X402, Skukuza, 1350, RSA

Although it is known that members of at least 36 species of African wildlife have succumbed after becoming infected with *Bacillus anthracis*[1], few accounts dealing specifically with the pathology of the disease in them, appear in the literature.

The opportunity arose during the recent outbreak of anthrax in the Kruger National Park, to conduct necropsies on a number of game animals that died naturally of the disease. Even though a large number of animals died during the outbreak (in excess of 1500 during each of two consecutive years) few carcases were available for examination due to the vastness of the game reserve, the presence of scavengers, and the rapid decomposition of carcases due to very high environmental temperatures: temperatures in excess of 40°C not being uncommon. A total of 18 of these animals were necropsied and, in addition, the findings of 9 impala used in a number of unrelated experiments dealing with the disease are included with that of the natural disease.

As in domesticated animals, a marked variation in the pattern of lesions encountered in the different species, characterized the disease. According to these findings, the various manifestations may be categorized as follows:

1. *Severe, peracute septicaemia* without organ localization (9 experimental cases; impala [*Aepyceros melampus*]). Essentially characterized by severe congestion, cyanosis, poorly-clotting blood, and no splenomegaly. Histologically severe brain oedema occurred consistently.
2. *Typical haemorrhagic septicaemia* [natural cases of the disease in kudu [*Tragelaphus strepsiceros*]) (4), nyala *(Tragelaphus angasii)* (1), waterbuck *(Kobus ellipsiprymnus)* (1) and roan antelope *(Hippotragus equinus)* (1)]. These animals manifest severe congestion, multiple petechial haemorrhages (sub-cutaneous and in serosae), cyanosis, poorly-clotting blood, a pronounced splenomegaly (most outspoken in the myala), and a severe, diffuse fibrinohaemorrhagic enteritis in kudu.
3. *Haemorrhagic septicaemia and variable organ localization* (natural cases in African buffalo [*Syncerus caffer*]). The lesions at necropsy vary from those in which few changes, except for severe congestion and cyanosis, occur, to those in which there are marked splenomegaly, pulmonary oedema, necrotic lymphadenitis (in individual lymph nodes), necrotic adrenalitis, and segmental, acute, necrohaemorrhagic enteritis.
4. *Localized lesions with no or late-onset septicaemia* (African lion [*Panthera leo*]). Lesions, of variable severity but usually outspoken and localized on the face, in the oral cavity and the regional lymph nodes. The changes are characterized by localized necrotic glossitis or stomatitis to locally extensive necrotic cellulitis of the lips and the face accompanied by severe oedema causing excessive swelling of the head. No lesions characteristic of septicaemia were encountered at necropsy.

Histologically necrotic lesions were invariably associated with tissue invasion by the bacteria. Thus in the case of the impala, scant necrosis only occurred histologically in few lymph nodes, whereas in those organs in the other species where necrosis was extensive, massive numbers of bacteria were encountered extravascularly.

The reasons for the death of animals in the various forms of the disease no doubt differ; whether they all die due to the effects of the toxin(s) is debatable. Particularly in the case of lions, dehydration and suffocation may play a primary role and a categorization other than that suggested by Lincoln *et al.* (1967)[2] may be considered in classifying susceptibility and expected response following infection.

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Pathology and diagnosis of anthrax in African wild dogs (*Lycaon pictus*)

*J. E. COOPER[1†], J. A. MATOVELO[2] and A. BASKERVILLE[3]

[1]Department of Medicine, [2] Department of Pathology, Faculty of Veterinary Medicine, Sokoine University of Agriculture, P.O. Box 3021, Morogoro, Tanzania, and [3] Salisbury, Wiltshire, UK
** (authors unable to attend the workshop)*

In 1993 African wild dogs *(Lycaon pictus)* in the Selous Game Reserve, Tanzania, showed evidence of ill-health. Within the affected pack three (17%) of 18 adult animals and 8 (33%) of 24 pups developed one or more signs of disease. Four (17%) of 24 pups died and two of these, designated pups 1 and 2, were obtained for *post-mortem* examination. Four other packs of dogs appeared to be unaffected.

Post-mortem examinations and supporting laboratory investigations were performed at the Faculty of Veterinary Medicine, Sokoine University of Agriculture, Tanzania. Both pups showed nasal haemorrhage, subcutaneous haematomata in the facial, sub-mandibular and thoracic regions; body cavities containing partly clotted blood; spleen swollen and markedly congested; lungs congested and oedematous; superficial lymph nodes markedly enlarged; stomach empty; small intestine inflamed; nymphs of *Amblyomma* spp in the inguinal region.

In addition, one pup showed skin ulceration, a necrotic gingival lesion, congestion of the serosa of the bladder, hyperaemia of the gastric mucosa and a cestode in the small intestine.

Laboratory investigations included aerobic bacteriology, histopathology and cytological examination of blood oedema fluid, lymph node smears and cut surfaces of organs. In addition, brain material was taken for rabies diagnosis and pieces of muscle were frozen for DNA studies.

A diagnosis of anthrax was made on the basis of the clinical history, gross findings, histopathology and the detection of bacteria morphologically resembling *Bacillus anthracis* in blood and lymph smears from pup 1.

A number of features of this investigation warrant discussion, in particular the failure a) to detect *Bacillus anthracis* in smears from pup 2, and b) to culture *Bacillus anthracis* from either carcass. Environmental factors, delay in examination and the type of diagnostic specimens taken may all have contributed.

The African wild dog has declined markedly in recent years[1] and infectious diseases are believed by some scientists to be a significant factor[2,3]. While rabies, canine distemper and certain other viral, rickettsial and protozoal conditions have attracted particular attention, the possible role of *Bacillus anthracis* should not be overlooked. This is not the first report of anthrax in the African wild dog[4,5,6] but whether the disease plays a part in regulating populations is unclear. In this outbreak only a small proportion of the pack was affected, mortality was less than 50% in animals showing morbidity and there was no evidence of transmission between dogs. These findings suggest that, in the Selous Game Reserve at any rate, anthrax is unlikely to play a large part in controlling numbers of *Lycaon pictus*[7].

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† Present addresss: National Avian Research Centre, PO Box 45553, Abu Dhabi United Arab Emirates.

Virulence gene determinants

A. FOUET, J-C. SIRARD and M. MOCK

Laboratoire de Génétique Moléculaire des Toxines, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris cédex 15, France

The *Bacillus anthracis* virulence factors that have been well characterized thus far are the three toxin components and the capsule. The toxin genes are localised on pXO1, a 185 kbp plasmid, and the capsule operon on pXO2, a 95 kbp plasmid.

In order to study the virulence with a genetic approach, specific tools have been developed: a suicide vector lacking a gram-positive origin of replication[12] has been used both for deleting various genes, and for constructing *B. anthracis* strains harbouring transcriptional fusions on pXO1. The deleted mutants resulted from double cross over events, whereas transcriptional fusion recombinants resulted from a single cross-over event between the regulatory region present on the recombinant plasmid and the same region present on pXO1 at the original locus.

The synthesis of the three toxin components and of the capsule is dependent on the presence of bicarbonate/CO_2. Results obtained with our fusion strains strongly suggested that the three toxin genes are coordinately regulated by bicarbonate and temperature[11]. The two virulence plasmids have been shown to each encode a transactivator, affecting the bicarbonate/CO_2 dependent synthesis of these virulence factors[8, 13, 14].

In the absence of the capsule, the cell wall of *B. anthracis* appears layered and is composed of small fragments displaying a highly patterned ultrastructure with a P6 or P1 symmetry[4, 6, 7]. S-layers can have morphogenetic functions, act as molecular sieves, or have protective functions, and have been shown, in some cases, to be virulence factors. A major component of this S-layer has been characterised. It is chromosomally encoded, and the gene *(sap)* has been sequenced. The deduced amino acid sequence indicated that the protein contains three putative "S-layer homology" sequences[9] in its N-terminus[2]. The gene has been deleted, and the mutant is devoid of an S-layer. The wild-type and mutant strains will be analysed in order to define the function of this S-layer. This gene seems to be less expressed in a pXO1$^+$ than in a pXO1$^-$ background (our observation and that of Farchaus *et al*[4], and the expression appears to be completely lost after many subcultures in the laboratory. The regulation of this S-layer component synthesis will be further studied.

Since pXO1 harbours the toxin genes, we decided to analyse this plasmid. A type 1 DNA topoisomerase gene (*topX*) was located on this plasmid[5]. In order to assign a role to *B. anthracis* plasmidic topoisomerase, a deleted strain was constructed and studied. Our results suggested that the stability of pXO1 in the presence of other plasmids is dependent on this *B. anthracis* topoisomerase, or on a complementary mutated gene product. DNA supercoiling has been implicated in the regulation of virulence gene expression[1]. Whatever the precise role of the *B. anthracis* plasmidic topoisomerase, either in the maintenance of, or in the expression from, the virulence plasmid, such properties would make this topoisomerase a fundamental factor involved in *B. anthracis* virulence.

However, other factors, than those already described, are probably implicated in *B. anthracis* virulence; preliminary results indicate that these can be found on all *B. anthracis* genetic elements, i.e. both virulence plasmids and the chromosome.

Pezard *et al.*[10] have shown that when *Bacillus subtilis* or *B. anthracis* spores were inoculated in the mouse footpad, there was a striking difference between *B. subtilis* on one hand, and all *B. anthracis* strains, even one cured for both virulence plasmids, on the other hand. The *B. subtilis* strain was eliminated very quickly whereas the *B. anthracis* strains persisted. This indicated that *B. anthracis* chromosome encodes products favouring *B. anthracis* survival in the host, i.e. virulence factors. However when mice were inoculated subcutaneously with various *B. anthracis* strains, differences were observed; pXO1$^+$ strains, even those where two toxin components were deleted, developed, whereas pXO1$^-$ strains did not. This suggested that pXO1 encodes factors necessary for *B. anthracis* to multiply in the host.

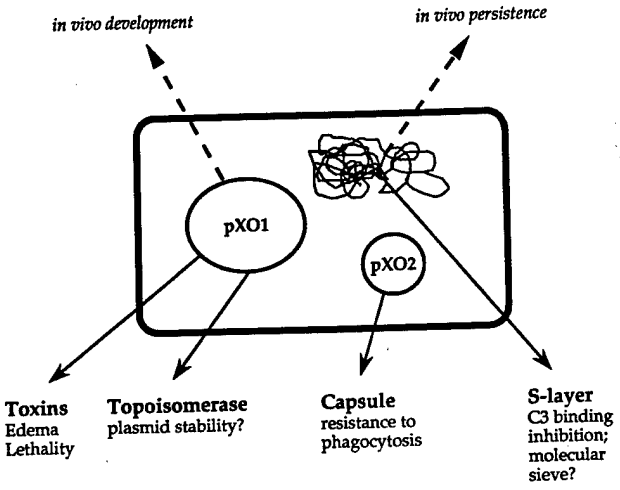

Figure 1. *Bacillus anthracis* virulence factors

Different approaches can be used to search for yet unknown virulence factors For instance, one can make the assumption that proteins which are synthesised *in vivo* may be virulence factors. The proteins which induce the synthesis of antibodies must be present in the host, and are therefore good candidates for this role. Such is the case for EA1, chromosomally encoded, and EA2, which gene is on pXO1[3]. The role of these two antigens in virulence will have to be studied. Other approaches are conceivable, like comparing proteins synthesised by *B. anthracis* strains grown either *in vivo* or *in vitro*. Since *B. anthracis* Sterne strain has no target organ, it is difficult to isolate, for these experiments, the bacteria grown *in vivo*. A technique was therefore developed in the laboratory, where diffusion chambers were introduced intraperitoneously in guinea pigs, and inoculated; samples were then harvested regularly.

The analysis of *B. anthracis* virulence gene determinants is only beginning, and very little is known. However the recent developments of powerful genetic tools and new animal models should enable us to successfully pursue these studies.

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Anthrax toxin gene regulation

ZHIHAO DAI and THERESA M. KOEHLER*

Department of Microbiology and Molecular Genetics, University of Texas Medical School at Houston, 6431 Fannin St., Houston, TX 77030, USA

*Correspondence

Summary

Synthesis of the *Bacillus anthracis* toxin proteins is enhanced during growth in defined media in elevated atmospheric CO_2 concentrations. Plasmid pXO1 carries the structural genes for the proteins: *cya* (edema factor), *lef* (lethal factor), and *pag* (protective antigen). Another pXO1 gene, *atx*A, encodes a *trans*-activator of anthrax toxin synthesis. To investigate the role of *atx*A in toxin gene expression, we constructed an *atx*A-null mutant and examined toxin gene transcription in cells grown in air and in 5% CO_2. We found that CO_2-enhanced transcription of all three toxin genes is observed only in the presence of *atx*A. The *pag* gene possesses two apparent transcription start sites, P1 and P2; only transcripts mapping to P1 are decreased in the *atx*A-null mutant. The *cya* and *lef* genes each have one apparent start site for transcription; transcripts with 5' ends mapping to these sites are not detected in the *atx*A-null mutant. *Atx*A protein and *atx*A mRNA levels do not appear to differ in cells grown in air and in 5% CO_2.

Introduction

CO_2 may be a physiologically significant signal for virulence gene expression when *B. anthracis* invades mammalian host tissues. Capsule and toxin protein synthesis is induced when strains are grown in defined media in elevated (5% or greater) atmospheric CO_2 concentrations[5,6]. For all three toxin genes, the CO_2 effect is at the level of transcription[1,7]. Mutants harboring transposon-generated insertions in the *atx*A locus, located on pXO1, produce decreased amounts of all three toxin proteins. These mutants can be complemented by addition of *atx*A in *trans*[8]. CO_2-enhanced transcription of *pag* is dependent upon the presence of *atx*A. The *pag* gene has two apparent start sites for transcription, P1 and P2. The level of transcripts with 5' ends mapping to P1, but not P2, is increased during growth in elevated CO_2[4].

Results and discussion

Effect of atxA on transcription of toxin genes and comparison of promoter sequences.

Previous studies of *atx*A gene function have employed mutants harboring transposon insertions in *atx*A[4,8]. We constructed an *atx*A-null mutant, UT53, in which the *atx*A gene is replaced with the Ω*km*-2 element. As reported previously for a Tn*917-LTV3*-derived *atx*A mutant, RNA transcripts with 5' ends mapping to P1 were significantly decreased in cultures of the *atx*A-null mutant. To determine whether *Atx*A regulates the *lef* and *cya* genes at the level of transcription, and to map the 5'-ends of *lef* and *cya* mRNA transcripts, we performed primer extension assays, using RNA from UM44 (*atx*A$^+$) and UT53 (*atx*A$^-$) cells grown in air and in 5% CO_2. Single primer extension products were detected for each gene when RNA from UM44 grown in CO_2 was tested. The relative amounts of these primer extension products from cells grown in air were significantly less. No *cya* and *lef* primer extension products were detected when RNA from UT53 grown in elevated CO_2 was tested.

No obvious similarities with respect to sequence homology or the locations of the apparent initiation sites were noted in the DNA sequences of the toxin gene promoter regions. Unlike *pag*, the *cya* and *lef* genes each have only one apparent transcription initiation site. The 58-bp region of dyad symmetry in the *pag* promoter region, was not found in the *lef* and *cya* promoter sequences. Using *B. subtilis* consensus data, we were unable to predict -35 regions corresponding to the *atx*A-regulated start sites.

Expression of atxA in air and in 5% CO_2

As *atx*A is required for the CO_2-induced increase in toxin gene transcription, we reasoned that transcription of the regulatory gene itself may be affected by CO_2. Primer extension reactions were performed using RNA extracted from UM44 cultures grown in air and in 5% CO_2. In each case, one major apparent transcription start site 99 bp upstream from the translational start site of *atx*A was detected. There was no significant difference in band intensity of the primer extension products when the different RNA preparations were used, indicating that transcription of *atx*A is not affected by CO_2.

*Atx*A protein levels in crude extracts of *B. anthracis* cells were assessed using Western hybridization. The coding sequence of *atx*A was cloned into pET-15b such that synthesis of His.Tag-*Atx*A was under control of the T7 promoter. The recombinant protein was purified using affinity chromatography and used to generate rabbit anti-*Atx*A serum. Protein samples from cells grown in air and in 5% CO_2 were examined for relative amounts of *Atx*A cross-reacting material. No significant differences were observed in samples prepared from cells grown in the different conditions.

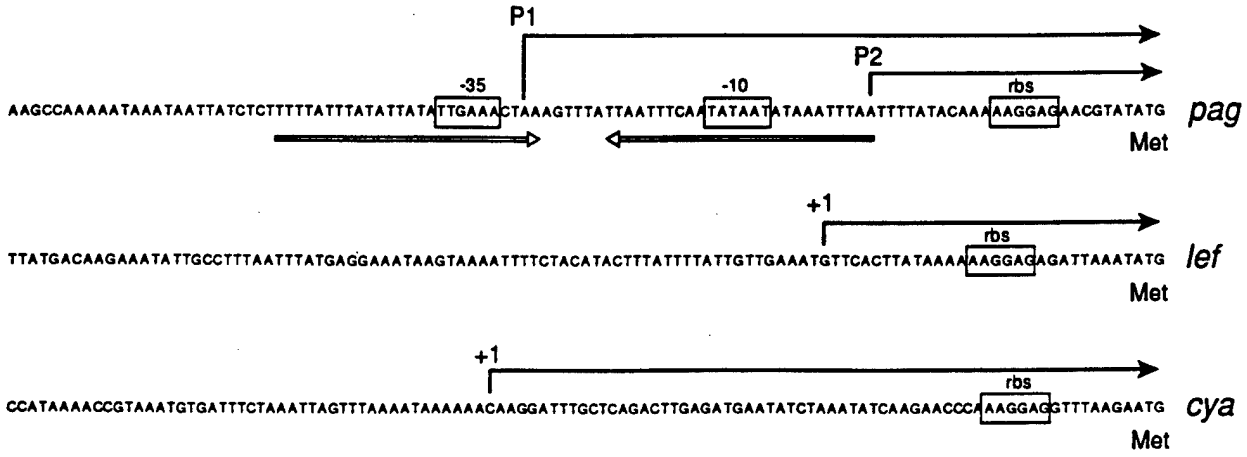

Figure 1. Comparison of the promoter regions of the toxin genes. The nucleotide sequences of the *pag*, *lef*, and *cya* genes, including the upstream sequences shown here, were reported previously[2,3,9]. Apparent start sites for transcription are indicated by solid arrows. Boxed sequences indicate predicted ribosome-binding sites (rbs) for all three genes, and -10 and -35 sites for P2 of *pag*. A region of dyad symmetry is marked by open arrows.

We can now view *pag*, *lef*, and *cya* as part of a regulon, in which transcription of all three toxin genes is activated *in trans* by the product of the same regulatory gene.

It appears that *atx*A is required for the CO_2 effect on toxin gene expression. Our results indicate that steady state levels of *atx*A mRNA and *Atx*A protein do not differ in cells grown in air compared to cells grown in 5% CO_2. It is possible that CO_2 affects function of the *Atx*A protein and/or expression of other gene products which may play a role(s) in toxin regulation.

Acknowledgements

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Role of chromosomally-encoded factors in virulence of *Bacillus anthracis* for mice and guinea pigs

A.S. STEPANOV, N.I. MIKSHIS and M.F. BOLOTNIKOVA

Laboratory of Isotope Analyses, Russian Research Antiplague Institute "Microbe", Universitetskaya 46, Saratov, 410071, Russia

The *Bacillus anthracis* tripartite toxin (Tox) and D-polyglutamic acid capsule (Cap) coded by the high molecular weight plasmids, pXO1 and pXO2, respectively, are the organism's only known virulence factors. Each is considered to play a critical role in the expression of the microbe's full pathogenicity for laboratory animals. However very little is known about other determinants which may also participate in the organism's virulence. The purpose of this study was to determine the a role of chromosomal loci in the full virulence of *B. anthracis* for mice and guinea pigs.

Studies in a mouse model

Using the *B. anthracis* strains 81/1, STI-1 and Pasteur and their isogenic derivatives cured of each ($pXO1^+pXO2^-$ and $pXO1^-pXO2^+$) or both ($pXO1^-pXO2^-$) plasmids, we demonstrated that the $pXO1^-pXO2^+$ strains remained lethal at low doses for outbred mice. The $pXO1^+pXO2^-$ derivatives were lethal for mice only if animals were challenged with high doses of living spores (10^7 - 10^8 cfu). Transductional transfer of the pXO2 plasmid from the high- and low-virulent strains into the $pXO1^-pXO2^-$ derivatives resulted in restoration of LD_{50} values typical of the fully virulent strains, thus indicating that plasmid-related virulence of *B. anthracis* for mice is mostly associated with products coded by the pXO2.

In order to analyze the involvement of chromosomal genes in the organism's virulence for mice, we compared amino acid requirements for growth of 48 strains of *B. anthracis* and 24 strains of closely related species. All the *B. anthracis* strains tested were found to be completely dependent on methionine in combination with one or two other sources of nitrogen. Among the strains of *B. cereus* and *B. thuringiensis*, however, some were found able to grow on the synthetic medium containing only salts and glucose. One such strain, *B. cereus* 569RA ($Apr^+Tg13ant^sPen^r$), was used as the donor of Met^+ locus in CP54*ant*-mediated transductions with the *B. anthracis* STI1ΔT4 ($Met^-Cys^-Tre^-Apr^-Tg13ant^rPen^s$, $pXO1^-pXO2^-$) used as the recipient. Among Met^+ transductant clones were isolated some which were characterized as $Met^+Cys^+Tre^+Apr^-Tg13ant^rPen^s$. Acquisition of the pXO2 by both Met^+ and Met^- strains led to at least a 10-fold increase of LD_{50} for mice challenged with the Met^+ derivative. Thus, loci located within or closely linked to Met^- region of the *B. anthracis* chromosome carry gene(s) responsible for the organism's virulence in mice.

Studies in a guinea pig model

The study showed that among seven *B. anthracis* strains, harboring both plasmids, four were 10- to 1000-fold less virulent for guinea pigs. To study the role of chromosome- and plasmid-borne differences in *B. anthracis* virulence for guinea pigs, the strains 81/1 and Pasteur were used as the representatives of high- and low-virulence strains, respectively. The reciprocal exchange of one or both of the homologous plasmid(s) between plasmid-free and monoplasmid derivatives of these strains revealed evidence that both plasmid- and chromosome-borne loci are responsible for strain-related differences in virulence of the organism for guinea pigs.

Using a novel extracellular protease assay procedure, we demonstrated that high- and low-virulence strains of *B. anthracis* differ in respect to extracellular protease activities. All highly virulent strains tested were able to degrade proteinaceous substrates including bovine and human serum albumins, casein, and fibrinogen. Less virulent strains of the microbe were characterized by markedly decreased activity against albumins, and slightly decreased ability to lyse casein and fibrinogen. To characterize the genetic event causing the inability of the less virulent strains to degrade albumins, mutagenesis of the strain Sterne $34F_2\Delta T$ ($pXO1^-pXO2^-$) with the transposon Tn*916* was used. Two low-proteolytic clones were isolated with phenotypes equivalent to the less virulent strains. Transductional transfer of Tn*916* from the mutants into the wild type strain Sterne $34F_2\Delta T$ resulted in acquisition of the donor strain phenotype. The results suggested the involvement in *B. anthracis* extracellular protease(s) synthesis of at least two loci which we designated as Apr (albumin protease) and Cpr (casein protease).

The similarity of phenotypic characteristics of the isolated mutants and the less virulent strains of *B. anthracis* demonstrates an important role of products coded by the chromosomal Apr locus or closely linked genes in virulence of the microbe for guinea pigs.

Extracellular proteases in *Bacillus anthracis*

[1]A.S. STEPANOV, [2]K.R. KLIMPEL and [2]S.H. LEPPLA

[1]Laboratory of Isotope Analyses, Russian Research Antiplague Institute "Microbe", Universitetskaya 46, Saratov 410071, Russia, and [2]Laboratory of Microbial Ecology, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892, USA

The virulence of *Bacillus anthracis* is primarily associated with syntheses of the tripartite exotoxin and the γ -linked poly-*D*-glutamic acid capsule, the structural and regulatory genes of which are located on the plasmids pXO1 and pXO2, respectively. Both of the virulence determinants are believed to contribute in part to the macrophage-related step in anthrax pathogenesis. Recent studies also indicate an involvement of chromosomally-encoded factors in *B. anthracis* virulence. However the nature of these factors as well as their primary target(s) remain obscure.

Our initial studies demonstrated 10- to 1000-fold differences in LD_{50} for guinea pigs among wild type capsule- and toxin-producing strains of the organism. The only distinctive characteristics shared by the strains of lower virulence was their inability to degrade albumin (Apr^-) in a substrate overlay assay and their substantially decreased ability to degrade other proteinaceous substrates. These characteristics are determined by chromosomal loci.

In order to identify loci involved in synthesis of extracellular proteases, we attempted to mutagenize the plasmid-free strains C1-1 and AS3 with the transposon Tn*917* (pTV1ts::Tn*917*, $Cm^rEm^rLm^r$). Em^rLm^r clones with altered ability to degrade proteinaceous substrates appeared in the libraries of Tn*917* insertional mutants with unusually high frequency, reaching 5-12%. Transductional transfer of the inserted antibiotic resistance marker with phage CP51*ts*45 into wild type strains resulted in acquisition of the proteolytically deficient phenotype in only 9-16% of the transductants. Control experiments performed with the wild type strains C1-1 and AS3 and their derivatives harboring the pTV1*ts* showed that these strains also formed phenotypically similar mutants with frequencies of 0.15-1.8 and 5-13%, respectively. This observation suggests the possible interaction of Tn*917*-encoded activities with *B. anthracis* chromosomal sequences so as to produce an unusually high level of mutants altered in proteolytic activity.

Further study of the phenomenon with the strain AS3 showed that spontaneous mutants formed by *B. anthracis* were relatively stable and could be divided into four phenotypically distinct groups, ranging from non- to superproteolytic. The isolated mutants, representing each of the above four groups, were also altered to different degrees in other chromosomally-encoded physiological characteristics, such as colony color and morphology, ability to sporulate and other characteristics considered to be virulence associated, including hemolysin activities, synthesis of yellow diffusible pigment, Congo Red sorption, resistance to lysozyme and a long chain formation.

Analyses of the supernatants of the isolated strains using SDS-PAGE zymography with gelatin as a substrate demonstrated a direct correlation of the amount and composition of the proteolytically active bands with each of proteolytically altered phenotype.

Experiments performed with RAW264.7 macrophage-like cells showed that the supernatants of the mutants were not toxic, whereas supernatants from the wild type plasmid-free strain killed the cells. In addition, a preparation of 102-fold purified extracellular protease (MW~87 kDa, pI~6.0) was also toxic to the macrophages.

Thus, the data indicate that chromosomally coded extracellular protease(s) of *B. anthracis* can be considered as a virulence factor which, like the plasmid-encoded toxin and capsule, participates in a macrophage-related step of anthrax pathogenesis.

The high frequency of appearance of mutants having complex alterations in chromosomally encoded virulence-associated characteristics in strains containing unintegrated pTV1*ts* may suggest a location of virulence genes within a locus of the organism's chromosome similar to "pathogenicity islands" described in other bacterial species. This process may be associated with a homologous mobile genetic element, and/or control/regulation by this element of the synthesis of a unique protein, that coordinately regulates the above characteristics. Current work includes a search for a locus responsible for the "instability" of extracellular protease-associated phenotypes, using a library of chromosomal DNA fragments inserted in the shuttle plasmid vector pHB201.

The influence of plasmid expression on electrokinetic potential of *Bacillus anthracis* cells

*S. YEREMIN and I. DYATLOV

Russian Anti-Plague Research Institute "Microbe", Universitetskaya Street 46, 410071 Saratov, Russia
** (authors unable to attend the workshop)*

The electrokinetic potential (EKP) characterises functional conductivity of the bacterial cell surface which to a great extent determines expression of their pathogenic characteristics.[1]

The aim of this study was to determine the pXO1 and pXO2 plasmid expression influence upon the EKP of *B. anthracis* cells. The EKP was determined by a modified amplitude frequency modified method[2]. We tested the *B. anthracis* cell EKP of virulent strain 81/1, and of its derivative strains lacking one or both of these plasmids. The results (Table 1) shows the decrease of ability to absorb to octane and increase of EKP, accordingly. *B. anthracis* strains harbouring pXO2 possessed low EKP and high ability to adsorb to octane.

When cells were grown in CO_2-rich atmosphere in the presence of bicarbonate, their EKP increased to 40mV and they lost any ability to adhere on octane, proving the hydrophilic nature of such cultures. Having a high level of EKP, the virulent strain *B. anthracis* 81/1 had a high hydrophobicity indicating the presence of adhesing structures on the cells' surfaces. The tetracycline resistance plasmid pBC16 in the cells essentially did not influence the cell-wall characteristics.

Table 1. Relevant characteristics of the *B. anthracis* strains tested

Strain	EKP	Adsorption to octane
81/1 (pXO1+, pXO2+)	-26.6±-0.3	23.3%
81/1 (pXO1+, pXO2-)	-25.9±0.4	8.5%
81/1 (pXO1-, pXO2+)	-17.9±0.2	27.8%
81//1 (pXO1-, pXO2-)	-22.9±0.3	23.6%

The confidence interval was determined for a probability of 95%.

B. anthracis STI-1 vaccine strain spores (pXO1+, pXO2-) had a charge of -18 mV. During the first hour of incubation at 37°C the growth of the spores was followed by an increase in the EKP. The total cell population could be divided into three clusters: ungrown spores, growing spores with the charge of -21.4 mV and short vegetative cells with -22.8 mV charge. After two hours of incubation at 36°C, nearly all the population consisted of vegetative cells; after three hours of cultivation the logarithmic growth of bacteria was followed by the growth of EKP up to -25 mV.

This result suggests that expression of *B. anthracis* plasmids influence greatly the structure of cell wall and the process of adhesion. The capsule's high charge and hydrophility confirm the importance of its functional meaning in the protection of *B. anthracis* cells from phagocytosis. The EKP cells potential measurement can easily reveal *B. anthracis* spores vitality.

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Macrophages are killed by the plasmid- and chromosomally- encoded factors synthesized by *Bacillus anthracis* inside and outside the host cell

A.S. STEPANOV and S.H. LEPPLA

Bacterial toxin and Vaccines Section, Laboratory of Microbial Ecology, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892, USA

The virulence of *Bacillus anthracis* is associated with the synthesis of lethal (LT) and edema (ET) toxins, coded by the plasmid pXO1, and the synthesis of γ-linked D-polyglutamic acid capsule, coded by the plasmid pXO2. LT kills macrophages and is lethal to laboratory animals when injected intravenously, whereas EF produces edema in tissues. The organism's capsule is believed to protect the bacilli against phagocytosis. Recent *in vitro* studies suggest that the interaction of LT and mouse macrophages plays a central role in causing the severe anthrax symptoms and lethality. However it remains unclear whether the toxin is synthesized by *B. anthracis* inside or outside the host cells *in vivo*. Chromosomally encoded factors are also known to participate in the expression of full pathogenicity of the organism for laboratory animals. However the target for their action on a cellular level is still unknown. The purpose of this study was to determine whether host macrophages are the target for both plasmid- and chromosomally-encoded virulence factors and whether the synthesis of these virulence factors by the organism occurs inside or outside host cells.

Our initial experiments demonstrated a rapid destruction of a macrophage monolayer as a result of addition of *B. anthracis* cells. To identify events leading to the monolayer disruption, we studied the interaction of vegetative cells of plasmid-free ($pXO1^-pXO2^-$) and monoplasmid ($pXO1^+pXO2^-$ or $pXO1^-pXO2^+$) isogenic derivatives of *B. anthracis* strains UM23, NH and AS1/1 with the mouse monocyte macrophage cell line RAW264.7.

The study showed that exposure of the macrophage monolayer to the vegetative cells of *B. anthracis* strains UM23 ($pXO1^+$ $pXO2^-$) and C1-1 ($pXO1^-$ $pXO2^-$) for 3 hours resulted in a 60-62% decrease of eukaryotic cell viability, as measured with the MTT assay. Addition of anti-serum against the protective antigen (the binding component of LT) did not prevent the disruption of macrophages. Decreased viability of macrophages (18-22%), was also observed when the cells were exposed to bacterial culture medium filtrates, thus indicating that macrophages are at least in part affected by factors other than LT secreted by *B. anthracis* outside the host cells and also suggesting a major role of the phagocytosed bacteria in the decrease of the viability of the macrophages.

To study phagocytosis of *B. anthracis* by the RAW264.7 cells a novel assay procedure was developed that allowed quantitation of the number of intracellular bacteria. Using this procedure we demonstrated that macrophages were able to ingest each of the *B. anthracis* strains tested, with the exception of the $pXO1^+pXO2^-$ derivative of the strain AS1/1, which resisted uptake due to formation of unusually long chains during its growth. The strains were able to survive and grow to different extent inside the macrophages. The most rapid rate of intracellular growth was observed with the isogenic derivatives of strain AS1/1, and the least with isogenic derivatives of the strain UM23. An intermediate rate of growth was observed with isogenic derivatives of the strain NH. Within each group of isogenic strains, the most rapid rate of intracellular growth was observed for the $pXO1^+$ $pXO2^-$ derivatives. The $pXO1^-$ $pXO2^+$ strains grew less rapidly. The plasmid-free strains were characterized by the least intracellular growth rate.

As a result of intracellular growth of bacteria, macrophage monolayers were disrupted in 24-36 h, in direct correlation to the intracellular growth rates. Despite the demonstrated ability of *B. anthracis* to grow intracellularly and kill the macrophages, the host cells partially suppressed intracellular growth. The addition of a phagocytosis inhibitor, cytochalasin B, to the infected monolayer resulted in 8-12-fold increase of viable intracellular bacteria within 12 h of incubation.

These findings indicate that macrophages are the target for complex effects of both chromosomally- and the plasmid-encoded virulence factors. Chromosomally encoded determinants, starting to act extra- and intracellularly apparently at the initial steps of the disease, lead to the death of the macrophages. The plasmid-encoded LT and capsule, synthesized by the actively growing phagocytosed bacteria, under the defined conditions appear to mostly act intracellularly, dramatically decreasing the viability of the macrophages. These data and the observed inability of the macrophages to ingest the $pXO1^+pXO2^-$ derivative of the highly virulent strain AS1/1, demonstrate a crucial role of bacterial phagocytosis at the initial steps of anthrax pathogenesis, where phagocytes may serve as a reservoir for accumulation, multiplication and dissemination of the microorganism throughout the body, thus playing a role in establishment and a subsequent progression of disease. At terminal steps of the disease the accumulated and released toxins appear to play a primary role.

Structure and function of *Bacillus anthracis* capsule operon and the role of its expression products in anthrax pathogenesis

N. A. STARITZYN, A. N. NOSKOV, A. V. STEPANOV and A. P. POMERANTSEV

State Scientific Centre of Applied Microbiology, Obolensk, Russia

Introduction

Bacillus anthracis produces two known virulence factors: a protein exotoxin and a poly-D-glutamic acid capsule. These are each encoded by a large plasmid, designated pXO1 and pXO2, respectively[6,11]. A region on pXO2 has been identified which contains three open reading frames expected to produce three putative proteins. The proteins, CapA, B, and C, are presumed to be involved in capsule polymerization and secretion. These proteins are expected to be associated with the cell-surface. The *B. anthracis* capsule operon has been studied so as to understand the regulation of expression of the capsule genes and the role of the gene products in anthrax pathogenesis.In addition, detection of pXO2 DNA by hybridization or PCR has been used to identify virulent strains [7].

Although the anthrax capsule has a recognized role in preventing phagocytosis of the bacteria, the role of the three putative capsule-synthesizing proteins in the interaction with host tissues remains unknown. The induction of antibodies against these proteins may occur as part of the immune response to infection or vaccination. A role for antibodies to these proteins is suggested by the fact that protective immunity following naturally occurring infection exceeds that achieved by vaccination with the Sterne strain ($pXO1^+$, $pXO2^-$)[1]. Because the infecting strains differ from Sterne only in possessing pXO2, these data suggest a role of the capsule proteins in immunity.

The hypothesis that the capsule-synthetic genes play a role in immunity could not be tested directly by comparing strains having and lacking pXO2 because of the possibility that any immunity induced by $pXO2^+$ strains could be due to the capsule itself. Therefore we cloned the capsule operon, selecting a source strain different from the previous used Davis strain[5], and evaluated its structure, expression, and the role of the three expressed proteins in anthrax pathogenesis.

Methods

B. anthracis virulent strains H-7 and 81/1, and vaccine strains 71/12, Sterne, and STI-1 were from the museum collection of the State Centre of Applied Microbiology, Obolensk. Strains of *Bacillus thuringiensis, Bacillus cereus, Staphylococcal aureus, Francisella tularensis* were from the same collection.

Plasmids for screening of recombinant clones were extracted by the Birnboim and Doly procedure[3], and further purified by density gradient centrifugation[9] for use in cloning procedures and restriction analysis. Total DNA for dot-blot hybridization was extracted after SDS lysis with phenol-chloroform and precipitated with ethanol.

For cloning of the capsule operon, the pXO1 plus pXO2 plasmid mixture from a virulent strain was digested with restriction endonucleases *Sac*I and *Sal*I and cloned into the same sites in pUC19. *Escherichia coli* JM103 transformants carrying the capsule operon were identified by comparing *Bam*HI endonuclease restriction fragment profiles to the published restriction map[8].

Electrophoresis of DNA was carried out in 0.8-1.0% agarose gels. Dot-blot hybridizations with biotinylated DNA were done using the BluGene kit (Life Technologies, Inc.) and Hybond N membranes (Amersham).

Results and discussion

The capsule operon was previously cloned from the avirulent Davis strain by partial XbaI digestion and ligation into plasmid pHY300PLK[5]. Positive clones were identified as ones giving immunoprecipitate rings when grown on agar containing anti-capsule serum. The cloned DNA was then sequenced and characterized[5]. In the work described here, the virulent strain H-7 was used as a source of plasmid DNA. The mixture of pXO1 and pXO2 identified by their molecular masses was digested with endonucleases *Sac*I and *Sal*I, the resulting fragments were ligated to pUC19 digested with the same enzymes, and the mixture was transformed into *E. coli* JM103. Clones carrying the recombinant plasmid were selected based on restriction endonuclease *Bam*HI digestion profiles using the fact that the capsule operon is the only *Sac*I-*Sal*I fragment that contains three *Bam*HI sites[8,10]. The restriction digestion pattern of the recombinant plasmid pSP19/91 containing the capsule operon is shown in Fig. 1. Dot-blot hybridization of pSP19/91 showed a positive signal only when probed with DNA of virulent *B. anthracis* strains (Fig. 2).

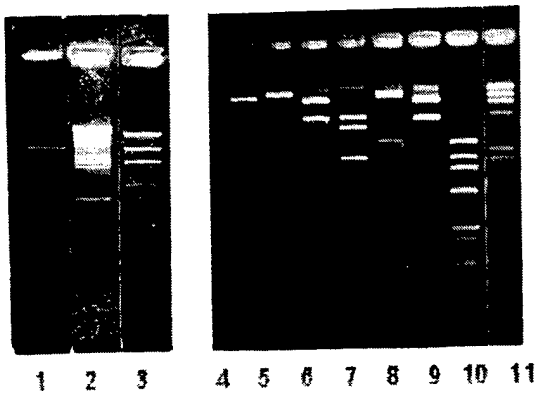

Fig 1. Restriction profiles of hybrid plasmid pSP19/91 with *B. anthracis cap* operon 1,4-pSP19/91, restricted with *Bam* HI; 3, 11-DNA of phage 1 restricted with *Hin*dIII, 5-10-pSP 19/91, restricted with *Sac*I, *Bg*lII, *Hin*dIII, *Sac*I+*Sal*I, *Sal*I+*Bg*lII, *Hin*dIII+*Eco*RI.

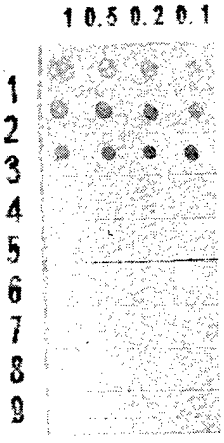

Fig 2. Hybridisation of plasmid pSP19/91 with DNA s of different sp. of microorganisms (The amount of DNA is on upperside in mcg)
1- *B. anthracis* CH-7, 2- *B. anthracis* 81/1, 3- *B. anthracis* 71/12, 4- *B. anthracis* Sterne, 5- *B. anthracis* STI-1, 6- *B. thuringiensis*, 7- *B. cereus*, 8- *S. aureus*, 9- *F. tularensis* A cole.

The strategy used for cloning, the restriction enzyme digestion pattern of the resulting plasmid in comparison to the map of pXO2 and the sequenced capsule operon[6,8,10], and the positive hybridization with DNA from virulent *B. anthracis* all indicate that the capsule operon was successfully cloned. Little expression of the proteins in *E. coli* JM103 is expected, although some indication of a capsule surrounding the colonies was observed microscopically.

Further work is underway to identify a system for expression of the capsule genes, so as to allow study of their regulation and the purification of the expressed proteins. Isolation of the *Cap* A, B, and C proteins will permit evaluation of their role in anthrax pathogenesis. The supposition is that these proteins will induce protective antibodies which prevent formation of the capsule by virulent cells growing in the host, thereby limiting the ability the *B. anthracis* to avoid phagocytosis.

Furthermore, the CO_2-dependent expression of the capsule synthesis genes suggests another mechanism by which antibodies may limit pathogenesis. The requirement for CO_2 suggests the presence of a sensor system[2], perhaps like that of the *Bordetella pertussis vir* genes[4]. The induction of antibodies against such a bacterial sensor system may be a basis of a novel concept for a new class of vaccines, one that inhibits critical physiological functions of the pathogen rather than secretion or action of classical virulence factors.

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Interaction of anthrax toxin with mammalian cells

STEPHEN H. LEPPLA[1], KURT R. KLIMPEL[1], YOGENDRA SINGH[1,2], VALERY M. GORDON[1] and NAVEEN ARORA[1,2]

[1]Laboratory of Microbial Ecology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892, and the [2]Centre for Biochemical Technology, Mall Road, Near Jubilee Hall, Delhi 10007, India

The three proteins of anthrax toxin, protective antigen (PA, 82 kDa), edema factor (EF, 90 kDa), and lethal factor (LF, 90 kDa) interact in pairwise combinations to produce two toxic activities (11,12). PA binds to a unidentified receptor present on the surface of most types of mammalian cells (5), and is cleaved at a single site by a cell-surface protease, recently shown to be furin (9). The carboxyl-terminal 63-kDa fragment (PA63) remains bound to the cell surface. PA63, but not native PA, has a high affinity site to which either LF or EF binds. The complex then enters cells by endocytosis. Acidification of endosomes causes PA63 to insert in the membrane and to form an oligomeric structure which is believed to constitute a protein-conductive channel (13). LF and EF appear to utilize this structure to translocate to the cytosol where they express the catalytic activities that cause toxicity. EF is known to be an adenylate cyclase (10), and recent evidence strongly suggests that LF is a metalloprotease (8).

The complete structure of PA was recently solved by X-ray diffraction (C. Petosa et al, manuscript in preparation). The results shows that domain assignments previously made by study of proteolytic fragments and by mutagenesis are consistent with the actual structure. The amino terminal region, aa 1-167, is removed by furin cleavage and has no role in internalization. Residues 168-600 are believed to form the membrane channel which causes binding and translocation of LF and EF. This region is highly similar in sequence to the corresponding region in *Clostridium perfringens* iota toxin. The carboxyl terminal 135 aa contains the receptor-recognition domain (16).

The role of furin in activation of PA was discovered through a directed effort to identify the substrate specificity of the cellular protease. Residues 164-167, ArgLysLysArg (RKKR) were mutagenized by a cassette procedure to produce a family of sequences rich in Arg and Lys (9). Assay of the resulting proteins for toxicity showed that the minimum sequence needed for activity was RxxR. Cleavage occurs to the right of this sequence, so that the Arg residues are designated as -4 and -1 relative to the bond cleaved. This substrate specificity matches that of the recently-identified cellular protease furin. Recombinant furin most efficiently cleaved those mutants which are active on cells. This correlation and protease inhibitor data supported the view that furin is the cellular protease that activates PA. We and others extended this finding to show that the proteolytic activation of diphtheria toxin (17), *Pseudomonas exotoxin* (PE) (4,14), and Shiga toxin (6) also involve furin (7). Perhaps most convincing were studies with furin-deficient cultured cells, to be described below.

Structure-function analysis of LF and EF begins with the fact that these two proteins have sequence similarity within the first 250 residues. The clear implication that this is the PA-binding region was confirmed by linker insertion mutagenesis of LF (15). To define the exact region of LF required for translocation, fusions were made of varying portions of LF to the ADP-ribosylation domain (domain III) of PE (1). This analysis showed that residues 1-254 were sufficient to produce translocation, because a fusion of LF1-254 with PE-III was highly toxic to cultured cells, but only when PA is administered simultaneously (1). Similar fusions were then made to the catalytic domains of diphtheria toxin (2), Shiga toxin (2), and tetanus toxin (3). Each of these fusion proteins is toxic to particular types of cultured cells, showing that the membrane translocation function in PA is very efficient. Because the translocation mechanism can accommodate a number of different polypeptides, this system has potential for use in the development of therapeutic agents.

The highly toxic fusion proteins such as LF1-254-PE-III provide a convenient method for detection of functional PA receptors on animal cells. This fusion protein will kill any cell having the PA receptor, provided that a protease such as furin is available to activate PA. Of the many cultured cells tested in this way, only a few cell types were found to lack receptor. These include GG2EE, a macrophage line derived from C3H/HeJ mice by immortalization with the J2 retrovirus, and two human lymphoma lines, Raji and Daudi. Thus, only a few cell lines lack the PA receptor.

The fusion protein LF1-254-PE-III also provides a means to select somatic cell mutants altered in cellular components involved in toxin uptake or action. Chinese hamster ovary cells (CHO) were mutagenized with ethyl methane sulfonate, allowed to express their mutations for 4-5 days, and then selected with concentrations of PA + LF1-254-PE-III that killed >99.9% of the cells. Surviving colonies were screened with diphtheria toxin, PE, and the PA + LF1-254-PE-III combination to classify the mutations. CHO mutants having altered or inactive PA receptor, furin, or elongation factor 2 were obtained. The mutants altered in receptor were studied further. One cell line which had no detectable binding of ^{125}I-PA is designated CHO 9-2-1. Because this cell line has normal sensitivity to PE and DT, it appears that the receptors for these three toxins are distinct molecules. The mutant CHO 9-2-1 will be useful in genetic analysis designed to identify the receptor.

Other CHO mutants that were identified that appear to lack furin. These cells are completely resistant to PE and to PA mutants that have the cleavage site sequence ArgAlaAlaArg. The relative potencies of PA mutants altered at the cleavage site on wild type and furin-deficient CHO cells showed that furin is the major protease responsible for activation of PA, but that other cellular proteases can contribute to cleavage of native PA by recognition of the additional basic residues at the site.

Together, these studies provide a more complete understanding of how the toxin components interact with each other and with cells. This information will guide attempts to identify critical amino acid sequences which could be targeted with specific antibodies to block toxin action and help control *Bacillus anthracis* infections.

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Cysteine mutants of anthrax toxin protective antigen as tools to probe structure and function

KURT R. KLIMPEL and STEPHEN H. LEPPLA

National Institute of Dental Research, NIH, Bethesda, MD 20892, USA

Bacillus anthracis secretes two toxins into the extracellular medium during growth. The two toxins consist of three distinct proteins which combine in a pairwise fashion. Protective antigen (PA) can combine with lethal factor (LF) or edema factor (EF). PA combined with LF makes lethal toxin while PA combined with EF makes edema toxin. In a simple model of intoxication PA (83 kDa) binds to a protein receptor on the surface of cells. PA is cleaved by a cellular protease and the amino-terminal 20-kDa fragment is released leaving PA (63 kDa) bound to the cell. It is only this form of PA which is able to bind either LF or EF and translocate these proteins to the cytosol where they exert their effects[3]. Interestingly, each of the three mature anthrax toxin proteins lacks the amino acid cysteine. The reactive nature of cysteine make it an ideal target for various chemical reactions which would enable us to modify certain regions of the protein. We used site directed mutagenesis to replace approximately every 50th residue throughout the carboxyl 63-kDa portion of PA with a cysteine residue.

We used overlapping PCR to construct twelve distinct PA mutants each with a single cysteine residue (Table 1)[1]. Each of the twelve PA-cys mutants was expressed in *Bacillus anthracis* strain UM23C1-1 and purified from the culture supernatant according to previously described methods[2]. The mutant proteins were visualized after SDS-PAGE by staining with Commassie blue. Each of the mutants was greater than 95% pure, although each preparation appeared to be nicked to some extent at the furin and/or the chymotrypsin sensitive sites. It is apparent that some of the PA-cys mutant proteins form high molecular weight structures which may be protein dimers (Table 1). When the identical samples were run in the presence of a reducing agent, the high molecular weight structures were no longer visualized, demonstrating that there were indeed dimers of particular PA-cys mutants. The six PA-cys mutants which form protein dimers may indicate that the cysteine residues are exposed on the surface of the protein while the six PA-cys mutants which do not form dimers have their cysteine residues buried inside the protein.

The unique cysteine residue in each of the mutants has allowed us to design a number of experiments to define the functional regions and sites of molecular interactions on PA. It was originally our intention to use a chemical reaction to specifically cleave each of the PA-cys mutants and then to purify the respective defined cleavage products and assess each fragment for its ability to bind LF, block PA binding to the receptor, block or enhance oligomer formation as well as examine the fragments' role in other activities associated with PA. Unfortunately, many of the resulting fragments were insoluble and/or difficult to purify.

Each of the twelve PA-cys mutants were assayed for their ability to kill cultured cells as described previously[4]. The toxicity data are shown in Figure 1. PA-cys mutants F313C, T357C, S429C and S717C were all non-toxic. S248C and S278C had altered toxicity while S170C, T488C, T548C, S623C, S667C and G735C were all fully toxic. There is no apparent link between the degree of toxicity and the ability of each mutant to form dimers (Table 1). The frequency with which cysteine substitutions altered the toxicity of PA was unexpected. We hypothesized that the reactive nature of the cysteine residue was responsible for the inactivation and that the position of the residue in the protein was of secondary importance. We tested this hypothesis by chemically inactivating the cysteine residue in each of the mutants with N-ethylmaleimide (NEM). After treatment with NEM, mutants S248C, S278C and F313C each regained full toxicity (Figure 2). Inactivation of the cysteine residue in mutants T357C, S429C and S717C had no effect; these mutants were still not toxic.

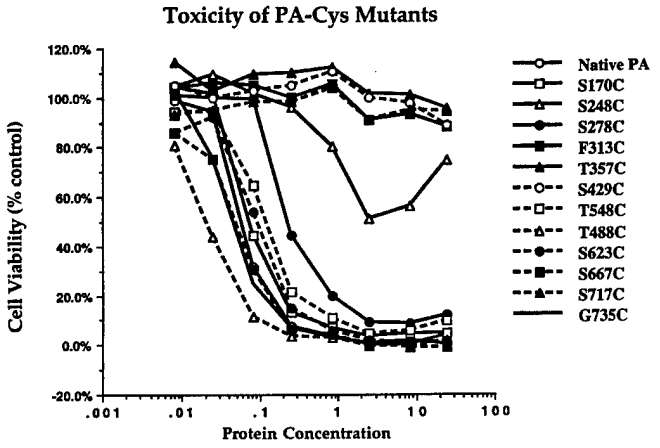

Figure 1. Toxicity of PA-cys mutants on RAW264.7 cells. LF was added at a constant amount (500 ng/ml).

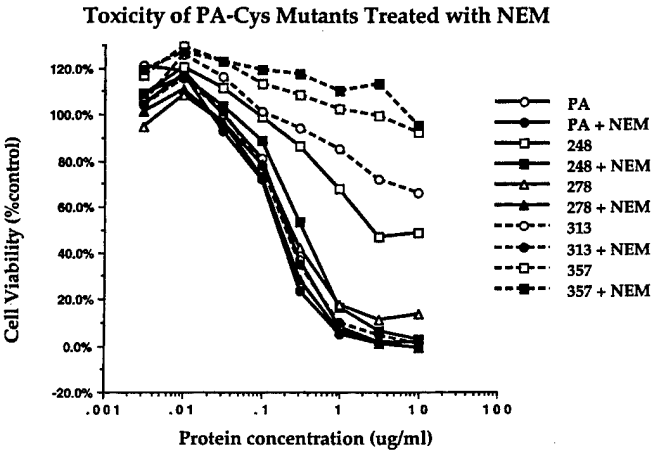

Figure 2. Toxicity of PA-cys mutants treated with NEM. The amount of LF was constant (500 ng/ml).

The protective antigen protein has a number of distinct functions. These include binding to the cellular receptor, binding to lethal factor or edema factor, forming an heptameric PA complex and translocating lethal factor or edema factor to the cell cytosol. Each of these functions can only be accomplished after modifications of the protein by cellular proteases or the cellular environment. The PA-cys mutants described here may begin to help us understand how this protein works to accomplish these feats.

Table 1. Activity of protective antigen cysteine mutants

Protein	Dimer Formation	Toxicity on RAW 264.7 cells
wild type PA	-	+
S170C	+	+
S248C	+	-/+
S278C	+	+/-
F313C	+/-	-
T357C	-/+	-
S429C	+	-
T488C	-	+
T548C	-	+
S623C	+	+
S667C	-	+
S717C	++	-
G735C	-	+

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The use of a regulated T7 RNA polymerase-based transcription system for the expression of the anthrax toxin and heterologous genes in *Bacillus anthracis*

DONALD L. ROBERTSON and FRANK SPANGLER

Department of Chemistry and Biochemistry and the BYU Cancer Research Center, Brigham Young University, Provo, Utah 84602, USA

Introduction

The goal of the experiments reported here has been to develop a set of expression vectors than can be used for the high-level expression of the individual anthrax toxin genes. This gene expression system has the potential to be valuable as well for expressing heterologous genes and for their secretion from the cell for easy purification. *Escherichia coli* and *Bacillus subtilis* have each been used for gene expression studies and are generally regarded as completely safe. In contrast, virtually no one, outside of a limited number of researchers who study *Bacillus anthracis*, has used *B. anthracis* for gene expression studies. In order to create a safer organism for protein expression, we also describe experiments which are necessary to produce an asporogenic *B. anthracis*.

When these studies were begun, the genes for each of the anthrax toxin components, edema factor (EF; *cya*), lethal factor (LF; *lef*) and protective antigen (PA; *pag*), had been cloned and sequenced (1-8). The high-level expression of PA in *B. subtilis* has also been reported, but neither LF or EF has been expressed at these high levels. The extremely high-levels of expression for the cloned PA gene in *B. subtilis* is probably due to the active pag promoter, although other promoters in the pUB110 cloning vector may also be driving its transcription. Previously, we had fused the *pag* promoter to the *cya* coding region, but had not been successful in achieving the high levels of EF expression that we desired. Since it is desirable to express each of the individual toxin proteins in the absence of the other toxin components, both for safety considerations and for being able to study the biochemistry for each the toxin proteins, we have developed the high-level expression system described below.

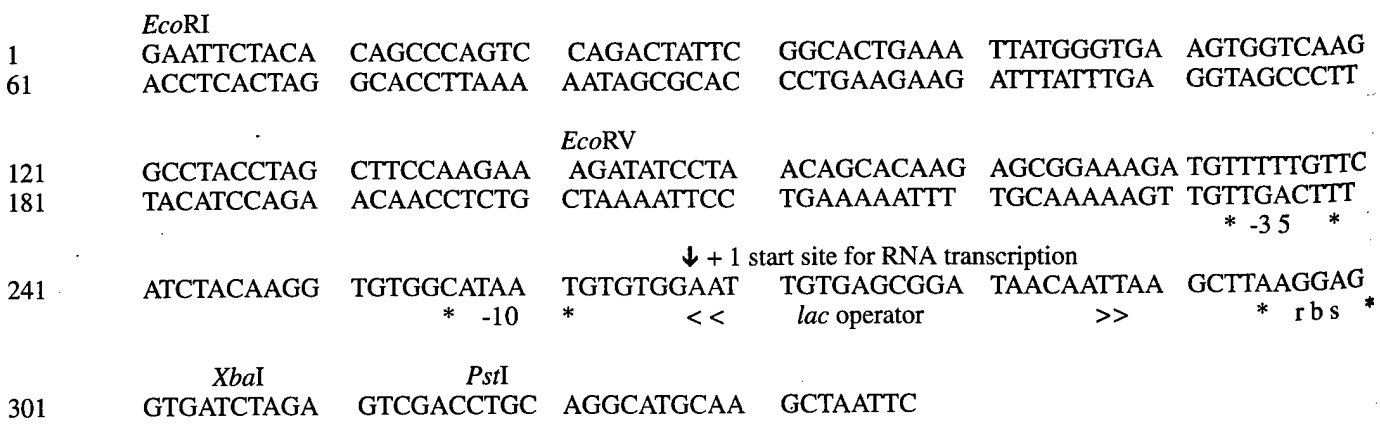

Figure 1. Nucleotide sequence for part of pSI-1 containing the *spac*-I promoter, the *lac* operator, the transcriptional start site and several restriction enzyme cleavage sites. This promoter region, along with the entire coding region for the *lac*I repressor gene, under transcriptional control of the penicillinase promoter, was transferred to P126 and was used in the integration vector as a single *Eco*RI-to-*Bam*HI DNA fragment. The T7 RNA polymerase gene was inserted between the *Xba*I and *Pst*I cleavage sites to produce P126-TL.

The approach that we employed for the high-level expression of the anthrax toxin genes involved the construction of a plasmid containing the T7 RNA polymerase gene (*T7gene1*) downstream from the inducible promoter found in pSI-1 (11,13,14). Figure 1 shows the nucleotide sequence for the region of pSI-1 which contains the strong spac-1 phage promoter. This promoter region as well as the lac repressor, cloned downstream from the penicillinase promoter, was removed from pSI-1 as an *Eco*RI-to-*Bam*HI DNA fragment and cloned into P126 (see the legend of Figure 2 for description of this plasmid).

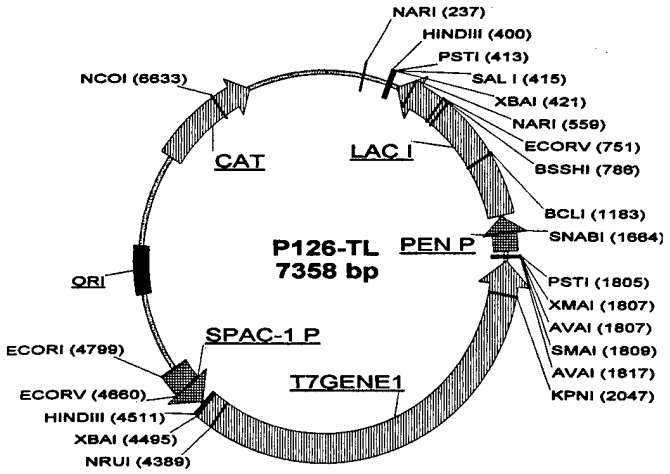

Figure 2. Plasmid P126-TL is derived from pUC12 and has the CAT gene from pC194 inserted into the *Dra*I cleavage sites of pUC12, resulting in the removal of the ampillicin resistance gene. The T7 RNA polymerase gene has been inserted downstream from the *spac*-1 promoter from pSI-1 (see Figure 1). In addition, the *lac*I repressor gene is transcribed from the penicillinase promoter so that the repressor is produced at all times. The unique *Sma*I cleavage site is the location where DNA required for integration has been inserted.

The T7 RNA Polymerase gene, which was originally isolated from pAR1153 (9,10), was inserted into the *XbaI* and *PstI* cleavage sites of the pSI-1 cloning region had previously been transferred into P126. The *T7gene1* DNA, from pAR1153, was subcloned into pBluescriptSK+ in an orientation so that the *PstI* recognition site in this plasmid was located downstream from the 3'-end of the *T7gene1* protein open reading frame. Then, we used site-specific mutangenesis procedures (18) to introduce a *XbaI* cleavage site just upstream of the normal translational start codon so that when inserted into the cloning region of pSI-1, the strong *Bacillus* ribosome binding site present in pSI-1 would be used for translation of T7 RNA polymerase. Subsequently, the *T7gene1* was inserted into plasmid P126 to produce P126-TL which can then be used for expression of T7 RNA polymerase.

Homologous recombination can be used to insert this DNA into the *B. anthracis* chromosome if genomic DNA is also present (12). Although we have not yet succeeded in inserting this expression plasmid into the *B. anthracis* chromosome, T7 RNA polymerase has been produced in *B. anthracis* using a self-replicating plasmid which contained P126-TL, but which also had the pUB110 replicon. Since the toxin gene expression plasmids also contained the pUB110 replicon, plasmid instability was observed even though the two plasmids contained the CAT gene and the kanamycin resistance gene, respectively. It is for this reason that integration of the *T7gene1* DNA is necessary for producing a stable expression system.

For insertion of the *T7gene1*-containing DNA (P126-TL) into the genome of *B. anthracis*, we used a piece of DNA specific for the *B. anthracis* chromosome. Initially, we decided to use a gene specific for sporulation as the homologous DNA required for integration (15-17). The use of this DNA would serve two purposes: (I) this DNA would be *B. anthracis* and (ii) integration of this plasmid into this gene within the *B. anthracis* genome would inactivate a sporulation-specific gene, rendering the sporulation pathway non-functional. At first, we did not have a sporulation-specific DNA from *B. anthracis* available. Using the assumption that sporulation genes are conserved in different *Bacillus* species, we attempted to use PCR (polymerase chain reaction) to isolate a gene from *B. anthracis* homologous to the *spo*0H gene of *B. subtilis*. This was accomplished using primers specific for a region of the *spo*0H gene which represents the sporulation-specific region, rather than the region which is homologous to all sigma (o) factors involved in transcriptional control. To this end, the 5'-end of the o30 transcription factor which is sporulation-specific and not transcription factor specific, was copied using PCR and used for hyridization to *B. anthracis* DNA. The assumption here was that the difference in GC% between *B. anthracis* and *B. subtilis* would likely be too great to use the *B. subtilis*-specific primers for PCR directly. A DNA fragment from *B. anthracis*, cloned in λZAPII, was isolated which was homologous to the *B. subtilis*-specific *spo*0H DNA. This DNA could be used for preparation of an integration vector. Alternatively, we also found the sequence of the *B. anthracis spo*0A gene, which is also absolutely required for sporulation to occur. Based on the published sequence of the *B. anthracis spo*0A gene sequence, which encodes a modulatory protein required for sporulation, we performed a PCR and this DNA was used in the construction of the integration vector. However, at the present time, we have not been successful in integrating the T7 RNA polymerase expression vector into several different *B. anthracis* strains using an integration vector containing either the spo0H or *spo*0A DNAs. It should be noted that DNA from *E. coli* can be transferred into *B. anthracis* only if the DNA has been isolated from *dam-* cells, since *dam-* methylated DNA is restricted in all strains of *B. anthracis* examined.

The plasmid that we used for the toxin gene shuttle plasmid is derived from pUBUC18, which serves as an efficient *Bacillus-E. coli* shuttle vector. This plasmid contains both *Bacillus* and *E. coli* replicons and was constructed using pUB110 and pUC18. The pUB110 DNA was cleaved with *Eco*RI and *Bam*HI (this DNA was then blunt-ended) and cloned into *Dra*I-cleaved pUC18. This plasmid contains the replication regions from pUB110 (*rep* protein, *ori*U, and *pal*U) and can be grown in the *Bacillus* species.

The multicloning region from pET21a, which contains the T7 RNA polymerase promoter, the T7 RNA polymerase terminator, a functional *E. coli* ribosome binding site (rbs) as well as several unique restriction enzyme cleavage sites, was cloned into pUBUC18 using several cloning steps, although these procedures are not described here. The resultant shuttle plasmid is designated pDR181 (see Figure 3, which shows the *cya* gene cloned into pDR181) and serves as the toxin gene expression vector. Each of the toxin genes has been inserted downstream of the T7 RNA polymerase promoter in pDR181.

In order to insert the toxin genes into pDR181, each of the *B. anthracis* toxin genes had to be mutated to produce a unique *Xba*I cleavage site located immediately upstream from each of their open reading frames, but including their own ribosome binding sites. After mutagenesis, the pag gene was inserted into pDR181 using *Xba*I and *Bam*HI cleavages. The *cya* gene was cloned as a *Xba*I-to-*Nde*I DNA fragment after cleavage by these enzymes. The *lef* gene was inserted as a DNA fragment produced by *Xba*I, followed by experiments which confirmed the correct orientation of the LF gene. The resultant toxin plasmids are designated pDR182, pDR183, and pDR184, and contain the genes for LF, PA and EF, respectively. A partial restriction map for pDR184 is shown in Figure 3.

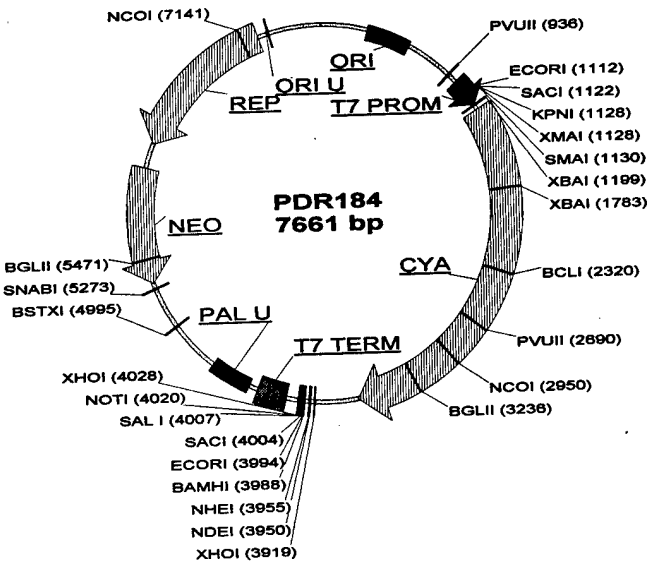

Figure 3. Anthrax toxin gene shuttle vector (pDR184) containing the *B. anthracis* edema factor *(cya)* toxin gene cloned into plasmid pDR181. Plasmids pDR182 and pDR183 contain the *B. anthracis* protective antigen *(pag)* and lethal factor *(lef)* genes, respectively. Each of these DNAs is derived from pDR181, which is derived from pUB110 and pUC18 (to produce pUBUC18) followed by the insertion of the polycloning region of pET21a, which contains the T7 RNA promoter and terminator.

The expression system described here consists of two unique components. First, the T7 RNA polymerase is under the regulatory control of the *lac* repressor so that the addition of IPTG will result in the expression of T7 RNA polymerase. This enzyme can then transcribe any DNA located downstream from a functional T7 promoter. The genetic components for this part of the expression system is integrated into the *B. anthracis* genome and bacteria containing this DNA can be selected by growth in chloramphenicol.

The second component consists of a DNA shuttle vector that contains one of the anthrax toxin genes. This plasmid contains the replication components found in pUB110 and is maintained in the presence of either neomycin orkanamycin. The T7 promoter, as well as the T7 terminator, flank the protein coding region for each of the toxin genes. The addition of IPTG results in the production of T7 RNA polymerase which then transcribes the toxin genes. Once translated, the toxin protein will be secreted from the cell. In a similar manner, this expression system can be set up to function in other *Bacillus* species as well. We have succeeded in using this gene expression system to produce heterologous proteins such as human ferritin.

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Analysis of Tn917 transposition in *Bacillus anthracis*

TIMOTHY HOOVER, SAMUEL CHATMON and ARTHUR FRIEDLANDER

U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21702-5011, USA

The current licensed U.S. human vaccine against *Bacillus anthracis* consists of aluminum hydroxide-adsorbed crude culture supernatants containing protective antigen (PA). Efforts at USAMRIID and elsewhere are underway to develop an improved vaccine that would overcome some of the problems associated with the current vaccine, which requires booster injections and causes occasional pain and swelling at the site of injection. The utility of a live spore preparation of non-encapsulated strains of *B. anthracis* in vaccinating livestock and humans suggests that an improved vaccine might consist of a live *B. anthracis* strain engineered to express either PA only, or PA with reduced expression of lethal factor (LF) and edema factor (EF). Furthermore, expression of foreign antigens in such a strain might stimulate a protective immune response against challenge from other pathogens, resulting in a multivalent vaccine.

B. anthracis is amenable to genetic manipulation. Various plasmids have been introduced into encapsulated and non-encapsulated strains and appear to replicate in a stable fashion under laboratory conditions. Live bacterial vaccine vectors, however, have suffered from plasmid instability after infection in mammalian hosts. Integrating foreign genes into the bacterial chromosome would overcome replicon instability, but reducing gene copy number might cause an unacceptably low level of antigen synthesis. Inserting antigen genes into highly expressed transcriptional units should allow for sufficient antigen synthesis and result in enhanced genetic stability. In this study we attempted to identify such transcriptional units and to examine a number of promoters for consensus sequences and/or structures.

The plasmid vector pLTV1 contains a temperature-sensitive origin of replication, a modified version of Tn917 designed to generate *lacZ* fusions, and several antibiotic markers. Copies of Tn917 integrated into host genomic DNA by transposition can be recovered in *E. coli* as recombinant plasmids because of the presence of the ColE1 origin within the transposable element. DNA flanking the site of insertion is included in the recovered plasmids and can be sequenced and possibly identified through database searches. Comparing beta-glactosidase activity among isolates harboring lacZ fusions would identify candidate genetic loci for expressing foreign antigens, and corresponding recovered genomic fragments would provide copies of *B. anthracis* DNA that could be further manipulated for the purpose of inserting antigen genes into the bacterial chromosome by homologous recombination.

Analysis of Tn917 transposition from pLTV1 into the genome of *B. anthracis* delta-Ames ($pX01^-$, $pX02^+$) showed the transposition frequency to be about tenfold less than the value reported for *B. subtilis* of 10^{-5} events per cell generation. Genomic preparations of chloramphenicol-resistant isolates cured of pLTV1 were digested with the restriction endonuclease EcoRI and circularized with T4 ligase. Aliquots were analyzed by inverse PCR. The resulting fragment sizes suggest that Tn917 transposition in this strain occurs in a highly random fashion. DNA sequence analysis of the recovered plasmids furthermore indicates no specific DNA sequence preference at the sites of insertion.

We recently obtained several hundred clones that had lost pLTV1 but now harbor *lacZ* fusions in regions transcribed during growth at various temperatures on LB plates containing chloramphenicol (10 µg/ml) and the chromogenic, beta-galactosidase substrate X-gal. DNA sequence analysis of the sites of integration of an initial group of 28 independent clones expressing *lacZ*, in contrast to non-expressors, revealed an apparent preference for insertion into pX02. Thirteen clones contained Tn917 insertions within a single locus that was homologous to other bacterial plasmid replication genes and shown to be linked to pX02 by PCR analysis. Nine other clones were likewise found with Tn917 in discrete sites of open reading frames related to genes involved in plasmid replication. Only six clones had Tn917 inserts in unknown open reading frames. The preference of Tn917 for pX02 in the process of selecting *lacZ* fusions has suggested that a $pX01^-$, $pX02^-$ strain would improve the likelihood of obtaining a broader spectrum of chromosomal insertions of Tn917.

The properties of pLTV1, interestingly, have allowed the development of a procedure by which a foreign gene can be incorporated and expressed in *B. anthracis* without the requirement of a selectable marker, such as an antibiotic resistance gene, remaining in the genome.

The views, opinions and/or findings contained in this abstract are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

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Cloning of *ori* pXO1 and *pag* gene of *B. anthracis* in *Francisella tularensis*

*VITALII M. PAVLOV, VLADIMIR M. TEDIKOV and ALEXANDER N. MOKRIEVICH

State Scientific Centre of Applied Microbiology Researches, Obolensk, Moscow Region, Russia
**(authors unable to attend the workshop)*

Summary

Bacillus plasmids can be replicated in the gram-negative micro-organism *Francisella tularensis*. It may be used for cloning large fragments of DNA which are not stable in *Bacillus* species. As a demonstration of this approach, the genes PA and *ori* pXO1 of *B. anthracis* were cloned in this microbe by a one-step procedure. Preliminary experiments showed that the vaccine strain of *F. tularensis* with *pag* can induce protection against both infections.

Introduction

One of the problems in research on *Bacillus* species is instability of hybrid plasmids, particularly with large fragments of heterologous DNA. This may be overcome in some cases by cloning this fragment in *Francisella tularensis*. We found earlier that *Bacillus* but not *E. coli* plasmids can be effectively replicated in this gram-negative micro-organism[2]. Monomeric forms of hybrid plasmids constructed *in vitro* may be introduced in *F. tularensis* by cryotransformation without the time-consuming procedure of preparing oligomeric forms in shuttle vectors. It is known that such non-specific additives as *Corynebacterium ovis* and *Bordetella pertussis* can enhance the protective action of PA in chemical vaccines to level of live spore vaccines[1]; cloning PA in *F. tularensis* results in analagous adjuvant properties.

Materials and methods

Overnight culture of *F. tularensis* was harvested from plate cultures, washed in 0.2 M KCI and resuspended in 0.1 M KCI, 0.1 $MgSO_4$, 0.01 Tris-acetate buffer pH 8.0. 50 µl of cell suspension (final concentration $5x10^{10}$ cell/ml) were mixed with 5 µl of DNA (0.05-0.5µg) in TE-buffer and incubated at room temperature for 10 min before freezing in liquid nitrogen. After thawing at 37° cells was plated on special media[2] and after 2-4 hours they were replaced on media with appropriate antibiotics. Electroporation of *B. anthracis* was carried out as described by Bartkus and Leppla[3].

Results

Plasmid pXO1 from STI-1 was partially digested with *Hin*dill and transformed in *F. tularensis*. Plasmid pBR328 which was used as a vector cannot be replicated in this microbe which is why only clones with hybrid plasmids where *ori* replication of pXO1 was upstream of the *tet*-gene of pBR328 were selected. Several Tc^r clones have plasmids with DNA insertions of about 20 kb, but after transforming them in *E. coli* or *B. subtilis*, their sizes were greatly reduced. Some of these deleted variants lost determinants of antibiotic resistance and transforming activity in *E. coli* or *B. subtilis*. Plasmid pORI 2 and pORI 3, which can be replicated both in *E. coli* and *B. subtilis*, have DNA insertions of about 2.5-3 kb (Fig.1) with *Pst*I and *Bam*HI sites that coincide with data of Robertson[4]. They were introduced and isolated from strain STI-1 and STI-1 lacking pX01.

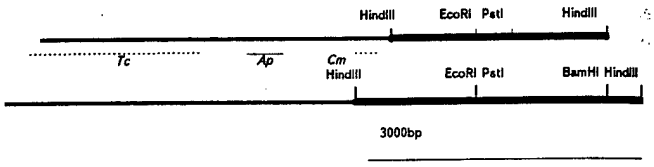

Figure 1. Restriction maps of plasmids pORI 2 and pORI 3. DNA from pXO1 is shown as the heavy line and from pBR328 as thin line. The region of *bla* and *cat* genes are deleted.

*Bam*HI fragments of pXO1 were also cloned in vector pFNL 200 (genetically-engineered derivative of cryptic plasmid pFNL 10 from *F. tularensis novicida* like. The strain with PA was selected using Ouchterlony gel diffusion. Experiments in golden hamsters showed some protective activity by this strain against anthrax and tularemia. (Marinin L. I. *et al.* unpublished data.)

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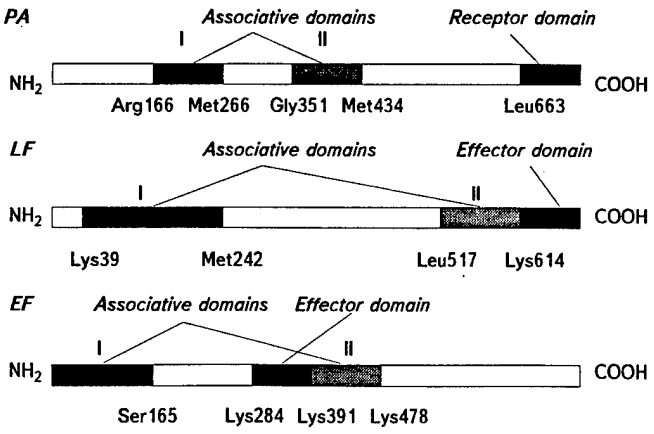
Elucidation of the roles of functional domains role in the molecules of anthrax toxin factors

*NOSKOV A. N. and KRAVCHENKO T. B.

State Scientific Centre of Applied Microbiology, Obolensk, Russia
**(authors unable to attend the workshop)*

Anthrax is an acute infectious disease, terminating with powerful toxic shock, caused by action of *Bacillus anthracis* exotoxin, a protein complex composed of subunit with receptor and associative functions known as protective antigen (PA) and effector subunits - lethal (LF) and edema (EF) factors (Stanley, 1961; Leppla, 1982, 1988).

For revealing of functionally-active domains in components of protein exotoxin we have applied a method of limited proteolysis and BrCN hydrolysis, essentially different from early applied insertion and deletion mutagenesis (Singh, 1989, 1991; Mock, 1988). PA, LF, and EF sets of fragments were prepared by using trypsin or minor quantities of *B. anthracis* proteases in purified preparations. Other fragments were prepared by BrCN hydrolysis. Almost all the fragments were separated by HPLC chromatography. The PA, LF and EF fragments were then used in concurrent inhibition tests of attatchment of exotoxin and its components to peritoneal macrophages. Then COOH-terminal amino acids and NH_2-terminal sequences were determined in functionally-active fragments. After this the arrangement of fragments in linear structure was determined from the known primary sequence of the components. Functionally-active areas in component molecules were revealed as the result (Figure).



It was found that the associative sites of all the components are constructed according to a uniform principle: the associative domains in the linear structure of molecules are located in two regions, thus intermolecular interaction of the first region was much stronger than that of the second (80% as compared with 20%). The receptor PA domain is located on the NH_2-terminal end in the linear molecule structure and shares a region of about 7 kDa. Removal of this domain leads to marked instability as reflected in an increase in protease sensitivity. This is in agreement with other authors (Figure; Singh 1991). This explains the high stability of the toxic complex when attached to a target cell. Advantages of a limited proteolysis method have been demonstrated for defining the clear functional domains instead of limited amino acid sequencing.

The presence of two intermolecular association areas in the structure of the components also results in greater stability of the protein components in solution and is an effective means of protection from bacterial and tissue proteases. In LF the biochemically active areas in effector subunits, are located at the COOH-terminal part of a molecule in a region of about 10 kDa. In EF this area is located between associative domains.

It was further established that penetration of not less than 500 molecules of LF are necessary to kill of one macrophage target cell. This represents extremely low LT cytotoxicity in comparison, for example, with diphtheria toxin, 1-2 molecules of which is enough for the same effect. It confirms that the main mode of exotoxin operation is by influence on the immune system by attacking macrophages. And, probably, this is the reason for low lethality of anthrax in comparison with diphtheria.

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Editor's note: The following article written by Dr Tikvah Alper, Max's wife for 62 years, was written towards the end of 1994 "for posterity and for those who are after me to write down some of the events in our lives, anecdotes they have heard from us".

Tikvah Alper was a distinguished scientist in her own right, initially in the field of radiobiology, but later with her main interest being the transmissible agent in scrapie and mad cow disease. Tragically, she died on 2 February 1995, but with Max's and their family's kind permission, we include this article in the proceedings as an entirely appropriate accolade to Max and his remarkable contributions to veterinary public health in the field of anthrax and several other diseases, and to Tikvah herself as his support for so many amazing years.

Development of the "Sterne Strain" of anthrax

T. ALPER

Max did not become a "professional" bacteriologist until 1947; but he worked as one from the time he was first appointed at Onderstepoort in 1934.

Onderstepoort was then, as now, a world-famous veterinary research institute, founded by Sir Arnold Theiler. Students of veterinary science in South Africa all attended Transvaal University College (later University of Pretoria), starting there, but doing the last three years of their training at the large farm and Institute, Onderstepoort, which is 8 miles north of Pretoria. Sir Arnold (Swiss in origin) was a magnificent teacher and a very hard taskmaster. There were usually about eight to ten students in each year, living in a hostel. The majority of the staff working at Onderstepoort also had homes on the site. The course was all-embracing, so graduates who subsequently got staff posts were expected to be able to tackle any line of work to which they were assigned. In the early 1930s effectively every veterinarian in South Africa was in the government service, either "in the field", serving farmers, or at Onderstepoort or one or two very small laboratories.

But after he qualified, in 1928, Max did not go into the veterinary service; he decided instead to take a job in what was then the Belgian Congo, to be in charge of an enormous cattle ranch. (His time there is another story, well worth telling!) He returned to South Africa after about 18 months and was appointed to a small laboratory just outside Pietermaritzburg, Allerton, where his sole duty, with several other young men of about the same age, was microscopic examination of smears sent in by field veterinarians in case the cattle had died of a dread disease known as East Coast Fever.

A morning spent examining smears was enough: afternoons were devoted by the young men to pursuits such as jigsaw puzzles. (Very occasionally there were "field" duties. Max was sent out once to solve a problem for a farmer, who wanted to express his gratitude for this free service by presenting Max with a case of whisky. He thought it would not be ethical to accept. But the Director at Allerton enjoyed his daily tot, and was furious with Max for his noble gesture!) M. was not satisfied with a laboratory life consisting of smear examination and jigsaw puzzles, so he amused himself by using the very meagre available facilities to learn something about the growth of bacteria in liquid culture. It was to continue this line of work at Allerton that Max and Tikvah (just married) set up a little laboratory, out of their own resources, in a "backyard room" meant for a servant's dwelling. (This is also a story which might be told in its own right!)

After about eighteen months at Allerton, Max applied for a transfer to work at Onderstepoort. His wish was reluctantly granted by the then Director, Dr. P. J. du Toit, *on the express condition that Max's nuisance of a wife would not demand to be allowed to work anywhere in the laboratories!* He was assigned to work under the Professor of Bacteriology, Eric Robinson, and one of his duties was the preparation of anthrax vaccine by the accepted method, based on that developed by Pasteur in the 1880s. This depended on reducing the virulence of anthrax bacilli by one means or another, for example heat treatment, so that injecting the treated bacilli would not kill the

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The Effects of Different Carbon Dioxide Concentrations on the Growth of Virulent Anthrax Strains.

Pathogenicity and Immunity Tests on Guinea-pigs and Sheep with Anthrax Variants derived from Virulent Strains.

By MAX STERNE, Section of Bacteriology, Onderstepoort.

4.—*To test the effect of different carbon dioxide concentrations on virulent strain* XXXIV.

This strain was isolated from the hide of a bovine seven days previously.

TABLE IV.

Tube No.	% CO_2.	Type of growth after. 24 hrs.	Type of growth after. 48 hrs.	Virulence Test. Subbed after. Hrs.	Virulence Test. Type of variant subbed.	Virulence Test. Death : Hours after inoculation. 20 40 60 80 100 200
A	0	—	*RS*			
B	0	*RS*	*RS*			
C	10	SM	*SM*	24	SM	† † †
		+++	++++	24	R (+++)	
D	10	*SM* ++	SM ++++			
E	20	*SM* +	SM +++			
F	30	*SM*	*SM*	24	*SM*	† † †
		++	+++	24	R (++)	
				48	*SM*	† † †
				48	R (+++)	†
G	30	*SM*	*SM*	48	*SM*	† † †
			+++	48	R (+++)	

Figure 1. Paste-up from Max's paper describing strain $34F_2$ for the first time.

animal but would make it immune to infection by a wild-type virulent strain. It was very difficult, however, to strike just the right balance between virulence and immunogenicity. One batch of Max's vaccine caused some deaths in inoculated cattle and, since farmers in South Africa formed a very powerful lobby, one farmer who had lost animals caused a complaint to be raised in Parliament. This was passed on to the Director at Onderstepoort, who, being aware of the problems involved, was decent enough never even to pass on the reprimand to Max! Quite a contrast with what happened in the Soviet Union at about this time, the time of vicious "purges". We learned that some makers of anthrax vaccine there had been executed for sabotage because a few of the injected cattle had died of anthrax.

Certain aspects of research on the anthrax bacillus suggested to M. that pathogenicity depended on its capsule; and he started playing with various methods that might give rise to mutated, uncapsulated strains. His chief, Prof. Robinson, told him that he should not waste his time on that nonsensical idea; but Max was stubborn, and Eric Robinson was very gentle, so M. continued with his search, isolating several non-capsulated strains, which he then tested for immunogenicity and virulence on small laboratory animals - excluding guinea-pigs at first, because no-one had ever managed to immunize a guinea-pig against anthrax. He isolated several strains that seemed to have something of the properties he was seeking, but then concentrated most of his further work on one of them. Being convinced that it was avirulent for every species on which he tried it, he thought that perhaps it might be possible to inject sufficient numbers of living spores into guinea-pigs so that they would indeed become immune. There were not many of the animals available, so he tried immunizing a group of only four. Then, after they might have acquired immunity, he injected each one of these, and each of a control group of four unimmunized animals, with sufficient virulent anthrax to have killed thousands of animals. If they were going to die of anthrax, they would do so within a few hours. This was such a critical and exciting experiment that Tikvah and Max (living at Onderstepoort at that time) got up at 2 am to see what had happened. There were the four immunized animals happily munching away at their greens; while the unimmunized ones were corpses. That was a tremendously exciting test of the complete non-virulence combined with good immunogenicity of what was much later to become known world-wide as the "Sterne Strain". (An interesting facet of that guinea-pig experiment was the comment made upon it by the examiner of Max's doctorate thesis. He said that, with only four animals in each group, the result was not statistically significant! As M. said, it was as if, having cut the heads off our animals, and leaving four intact, there was no statistically significant proof that cutting off an animal's head would kill it!)

Several circumstances combined to deny Max as much recognition, early enough, for this work as it merited. In those days and in far-off South Africa we were very innocent about such things. For example, Max's publications were only in the "Ondersterpoort Journal". This was only of specialist interest, and it took weeks and weeks to get to Europe and America. Such an important development in the field of public health should surely have been reported promptly in some much more widely read scientific journal, such as Nature, Science or even the Lancet: but Max himself, with a tendency to under-rate his achievements, would not have thought of doing this: nor, evidently, did it occur to Professor Robinson that some such publication should be sought. Of several papers published in the Onderstepoort Journal only one title so much as gives a clue as the fact that a completely avirulent, unattenuated strain was being used; and, to this day, those early papers of Max's that are still sometimes cited do not, in the titles, refer to the important features of the new vaccine strain.

In due course people read the papers in the Onderstepoort Journal and wrote to Max asking him to send them the strain, which he did quite freely. Just imagine this happening today, with every research Institute and probably most researchers seeking to patent every new biological entity capable of being patented! What is more, some people to whom Max sent cultures started writing about their work with them, without acknowledging its origin. This permitted the implication that the authors themselves had developed the strain.

As it happened, England declared war on Germany in 1939 precisely one day before Max and his family were due to leave for England, M. having been awarded a scholarship to do his Diploma in Bacteriology. His leave of absence was, of course, immediately cancelled. He might otherwise have found out much more promptly the truth about certain vaccine strains mentioned in the literature during the war years. He was very intrigued, for example, reading about the "Weybridge Strain", its origin not being mentioned in the relevant papers. It seemed so much like his, from the description given! Max finally did get to England in 1946, and in due course visited the Weybridge Laboratories to discuss anthrax with them. When he asked them how they had developed the "Weybridge Strain", they confessed that it was his - and seemed rather surprised that he had not realised it!

Farmers' attitudes towards the control and prevention of anthrax in Western Province, Zambia

DIETVORST, D.C.E.[1]

[1]*Coordinator Socio Economic Analysis Team, Livestock Development Project Phase II, Department of Veterinary and Tsetse Control Services, P.O. Box 910034 Mongu, Zambia*

Introduction

Since 1990, outbreaks of anthrax have occurred annually in Western Province. In response to the first reports of animal and human deaths, a large scale vaccination campaign was started in November 1990, and vaccination has since continued on a yearly basis. Although a considerable degree of control has been achieved, the pattern of outbreaks is persistent and spreading with a clear concentration of cases along the banks of the Zambezi river. Reports from veterinary field staff suggested that one of the factors which may contribute to sub-optimal control levels, is farmers' resistance to vaccination. Therefore, following a request by the Provincial Veterinary Officer, an assessment of farmers' views on anthrax and their attitudes towards vaccination was carried out. Between October 1993 and February 1994, farmers and veterinary field staff were visited in all four affected Districts of Western Province.

Results

Because of the importance of anthrax as a zoonose, vaccination is administered free of charge. However, limited availability of vaccine does not allow for a full vaccination coverage of risk areas and a portion of the vaccine is commonly kept in reserve to be used in case of outbreaks. This situation has led to cases where already infected cattle were vaccinated in the middle of an outbreak and have died practically upon being vaccinated. Farmers who had experienced such disasters, said they initially feared that it was the vaccine which carried the disease from kraal to kraal. However, practically all of them explained that they had later realised that those animals had already been sick and would have died anyway, and stated they would have their cattle vaccinated again.

Farmers' resistance to vaccination appears to be more closely related to the timing of vaccination campaigns. The ploughing season emerged as a very important element to be taken into account when planning a vaccination campaign. Anthrax vaccination campaigns have often been carried out during the ploughing season, which roughly extends from July to December, depending on the crop and the area. Farmers, when having their animals vaccinated, are advised not work their oxen for a period of two weeks, in order to allow the vaccine to distribute itself in the bloodstream and become effective. It was found that farmers take this advice seriously, and rather than risking to plough their oxen when this might have harmful consequences, they decide to withdraw them from vaccination altogether. Moreover, sometimes oxen are on loan for only a limited period and farmers cannot afford to rest them for two weeks if they also want to get their fields ploughed. This has serious consequences, since of all cattle, oxen move most frequently between kraals and camps, eg for purposes of ploughing or transport, and can easily carry the disease from one area to the next. This correlates with field staff responses on the history of anthrax in their catchment area. On several occasions the introduction of anthrax could be traced back to a particular span of oxen, that had travelled from a neighbouring area and came down with anthrax upon its arrival. Thus, for vaccination efforts to succeed, it is important that these are carried out prior to the ploughing season, ie before July/August.

Burying of anthrax carcasses generally meets with a lot of resistance among farmers. Most farmers bury the spleen, so as to get rid of the obviously affected part, but will eat the meat, despite the fact that veterinary staff tell them not to. Animals which have died of anthrax are often in good shape, since the course of the disease is fast and does not involve a gradual loss of condition. Burying an anthrax animal effectively means throwing away good food and forsaking the rare opportunity of a decent meal of meat. Secondly, the chances of acquiring anthrax through eating of infected meat are not very high. Even in cases where people had contracted the disease through the consumption of meat, farmers argued that many others had eaten from the same dish with no ill effect. Finally, farmers' knowledge of anthrax appears to be very limited. The majority of farmers only know anthrax by its enlarged spleen, can identify few other post-mortem signs, and cannot recognize the disease from an unopened carcass. Routes of transmission, both between animals and from animal to man, are practically unknown.

However, yet other factors, apart from the mere waste of food, make it virtually impossible to enforce such a practice. In Western Province, cattle are often kept by people who are not the actual owners, whether on long term *(mafisa)*, on temporary caretaking in the uplands *(bulisana)*, or on short term loans for ploughing or manuring *(kutulisa)*. In all these cases, the owner of the animal needs to be presented with its hide, and sometimes its head, when it has died while under caretaking (Figures). A condition which is strongly upheld as the animal's hide represents its identification. Thus, it is practically unavoidable that cattle which have died of anthrax are dragged to the village where they are skinned and slaughtered, or are butchered on the spot after which the fresh meat is carried to the homestead where it can be consumed or dried. Contamination of the soil is thus spread over long distances and brought in close proximity to human settlements.

Discussion

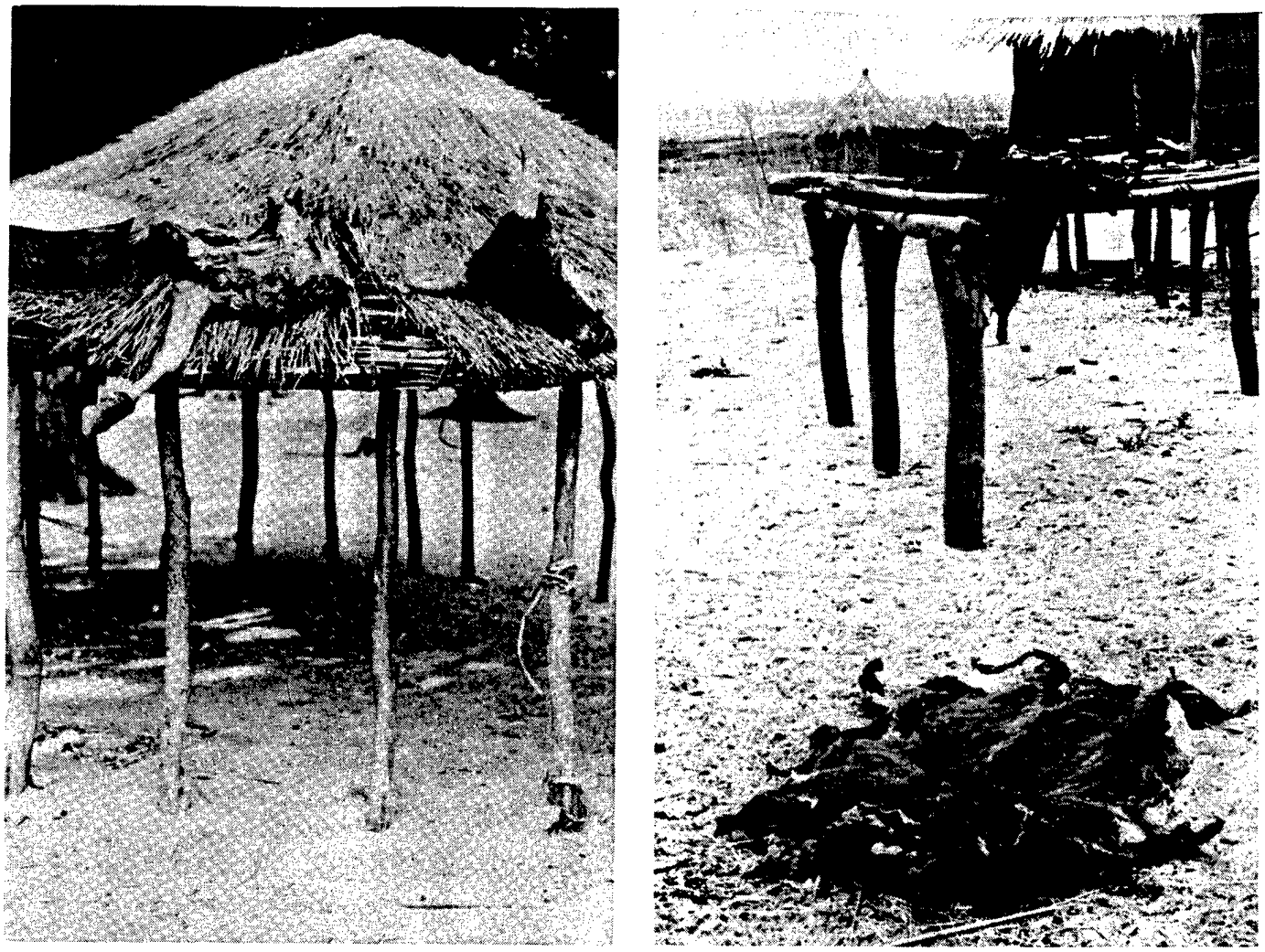
Strategies to control anthrax and prevent its spread normally include the adoption of widespread vaccination campaigns and the safe disposal of carcasses. As for the latter, under Western Province conditions, this is of limited value only. The prevailing custom of caretaking of cattle means that the opening of carcasses cannot be completely avoided.

Vaccination campaigns are a more reliable route to contain the spread of anthrax in Western Province. Farmers' resistance against vaccination, based on fear of introducing or spreading the disease through the vaccine, is not a major obstacle in the control of anthrax. It is a local problem only, occurring in areas where there has been no experience with anthrax before, and even there, attitudes tend to change over time. Apart from fear and ignorance, there are other reasons causing farmers not to vaccinate their cattle. Important among these is the pressure on oxen during the ploughing season. Farmers are told that oxen need to be rested for two weeks after vaccination, but some cannot afford to loose that ploughing time and decide to withhold their oxen from vaccination thus facilitating the

introduction of the disease from one area into another. Timely vaccination is of utmost importance in order to achieve acceptable levels of control.

Farmers' understanding of anthrax is an important ingredient in changing attitudes towards vaccination and other control measures. Farmers' knowledge of the disease appears very limited and the dissemination of information on anthrax to farmer level should be intensified.

From 1994, the timing of anthrax vaccination campaigns has been altered, taking into account seasonality of the disease, transhumance patterns, ploughing season and farmers' preferences. Extension material has been developed based upon findings from the assessment regarding farmers' views and practices. This material currently accompanies vaccination campaigns in the form of posters, booklets and a radio play (see following paper).



Figures. Caretaking. When an animal is being looked after on behalf of its absent owner, and it dies, the hide, and sometimes the head also, must be kept for the owner's return to prove its identity.

Editor's note

Following the previous International Workshop on Anthrax (11-13 April 1989, Winchester), the Veterinary Public Health unit of WHO formed a "working group on anthrax control and research". Seen by this group as one of the major components of anthrax control programmes was the element of education, particularly (but by no means exclusively) in endemic regions. In practice, it was proposed (WHO/CDS/VPH/91.98) that this should include preparation of educational material such as posters for public awareness, manuals for guidance at all levels from farmer/owner through health and laboratory workers, veterinarians and physicians, to ministry officials, and teaching kits, including instructional videos.

Because it would have been impractical to attempt to implement the overall control programmes proposed by the working group on a global basis all at one time, the idea of a "Model Country Programme" was conceived in which willing participants would implement the ideas and determine the optimal control procedures in practice.

In the event, even this proved over ambitious, but the Department of Veterinary and Tsetse Control Services, Western Province, Zambia, with the aid of the Dutch Government sponsored Livestock Development Project Phase II, took up the challenge in an exemplary manner, and, as part of the development of an educational programme on anthrax for the Province, produced posters and a book written in the local Silozi language.

The following story and pictures feature in the book and posters which are designed to convey in an intersting manner to those likely to encounter anthrax, the important points about the disease which they should be aware of. Radio programmes have also been produced using local people to act out the story.

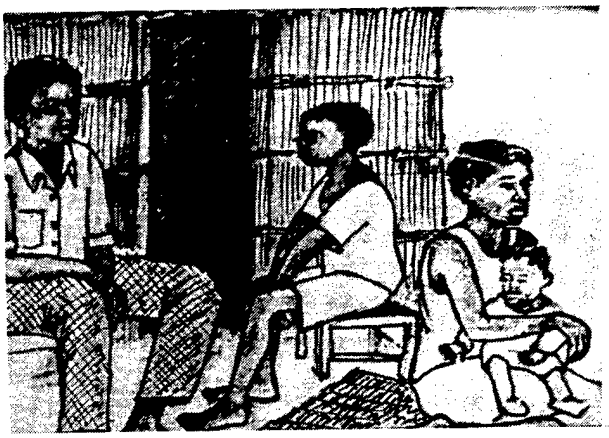
It is hoped that this model will be inspirational to other countries in their attempts to control, not only anthrax, but also other disease problems of major importance in their regions.

Educational material on anthrax for villages in western Zambia

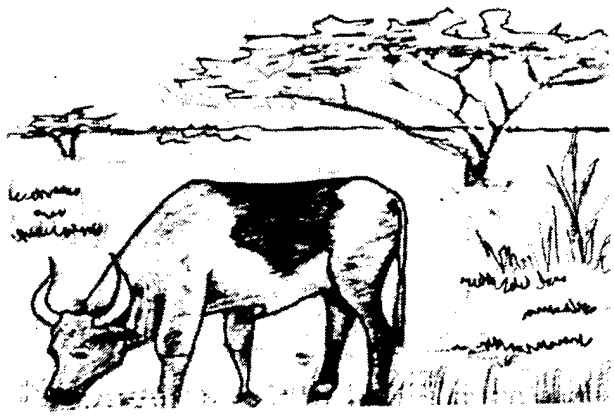
D. C. E. DIETVORST

Coordinator Socio Economic Analysis Team, Livestock Development Project Phase II, Department of Veterinary and Tsetse Control Services, P.O. Box 910034 Mongu, Zambia

Anthrax is a dangerous disease. Anthrax can kill animals, such as cattle, goats, pigs and dogs. More importantly, anthrax can kill even people. This is the story of Mr and Mrs Liseli and their two sons Likando and Sepiso.



Mr Liseli is a farmer. He keeps some 40 cattle. One day, one of his animals died of anthrax. Mr Liseli had not experienced the disease before and did not know what to do. Now he has learned, and his story is told here so that others can learn from his experience too.

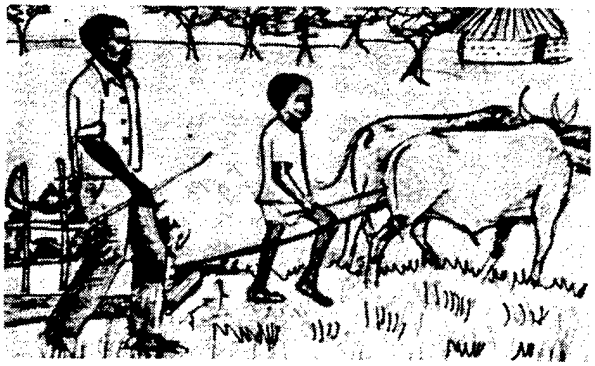


It is at the end of the dry season. Mr Liseli's cattle are grazing in the plain. The grass is short and cattle graze close to the ground. Last year in this area, some cattle had died of anthrax and their carcasses were opened. Along with the blood from these animals, anthrax spores seeped into the ground. Although the meat and hides of these animals were removed a long time ago, these spores are still on the ground, mixed with the soil. At this time, the grass is so short that the cattle are eating some soil too, while they graze. One of Mr Liseli's oxen is also swallowing spores which are mixed with the soil.

As soon as the spores are inside the body of the animal, they start multiplying themselves. Some 8 or 10 days later, the ox is found dead. Mr Liseli is surprised because it had looked normal that same morning, and showed no signs of illness. When animals are sick with anthrax, death usually comes suddenly. The bodies of these animals look bloated and sometimes a thick and dark looking blood comes out of their nose, mouth and anus. Also, the animals do not become stiff, even after they have been dead for some time.

Mr Liseli decides to cut up the animal because the carcass looks good, so it can be carried to the village. Mr Liseli and Likando work hard and after some time and ox' carcass has been cut into pieces. The meat looks fine and smells alright. Only the spleen is very big and filled with thick, almost black, blood. Mr Liseli tells his son to bury the spleen under the tree. Together they lift the meat onto the sledge.

What Likando and his father cannot see is that the blood of this ox is full with spores. The air around them is also filled with spores which are being released from the dead animal as it is cut into pieces. As they breath, these spores enter their body. More spores are smeared onto their skin along with the blood as they cut up the meat. The spores on the skin can enter the body through wounds and cuts on the skin. When anthrax spores enter the body of humans, they can cause serious illness and even death. The spores are drawn here to show where they are. But in reality is not possible to see them as they are too small.



Mr Liseli and Likando walk home with the meat heaped onto the sledge. After a few hours they approach their village. Some blood has fallen onto the soil, from where they slaughtered the oxen under the tree and all the way to their village. In each drop of blood there are anthrax spore which can make other animals sick when they swallow them together with soil while they graze. But because these spores are to small to be seen, Mr Liseli and Likando do not know about them.

As soon as they arrive home, Mrs Liseli prepares some of the meat for their evening dinner. The rest of the meat is cut into long strips and hung on a pole to dry, to be used later. The head and hoofs of the animal are stacked onto the pot rack to dry in the sun. Mr and Mrs Liseli do not know that in this way, the area around their house is also getting infested with anthrax spores, which fall onto the ground together with the blood dripping out of the meat.

That evening, Mr and Mrs Liseli and Likando enjoy a nice dinner, they eat nshima with meat. The meat tastes very good and they eat a lot. Sepiso, Likando's young brother, is still too small to eat meat. He is sitting impatiently on his mother's lap, waiting for milk. Because the meat is so nice, they do not think it is dangerous to eat. Especially because they are used to eating meat of animals that have died on their own and it has never made them sick. Unfortunately, anthrax is different and people can get very ill when they eat the meat of an animal that has died of anthrax.

After some days, Mr and Mrs Liseli fall ill. They have sores on their body. These sores are few, but very big. Mr Liseli has a large sore in his neck. It looks strange, roundish and thick, and has a large and slightly sunken black centre. His wife has a similar sore on her arm. Likando, the eldest son is even more ill. They do not understand what has made them ill and decide to go to the hospital.

The nurse at the hospital tells them they are infected with anthrax. She explains how spores can enter the body through the mouth or through the skin: through the mouth by inhaling air with spores or by eating meat from an infected animal. And through the skin by handling the carcass or meat of an animal that died from anthrax.

Likando has inhaled a lot of air with spores when he was cutting up the ox and while burying the spleen. His neck is very swollen and he breathes with difficulty. Mr and Mrs Liseli have developed sores on their skin because anthrax spores have entered their body; maybe while cutting and preparing the meat, or maybe while eating the meat. The nurse gives medicine to all three of them. At this moment, Likando is already too weak to stand and hangs limp from his mother's arms. She looks down at her son and is frightened that he may not recover.

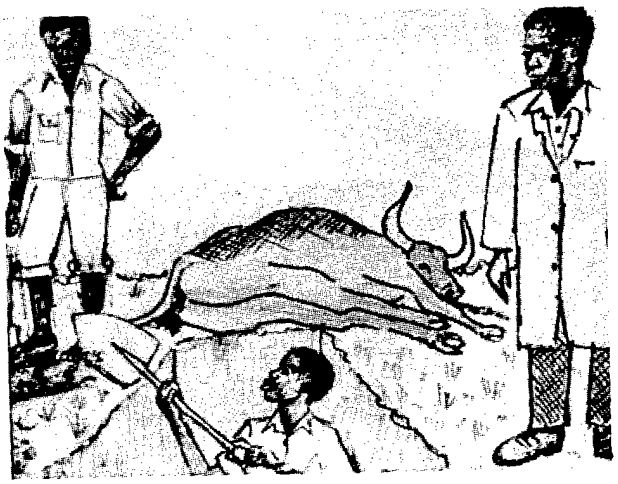
A few days later, Likando dies.

The nurse at the hospital did all she could to help him, but he could not be saved. His parents are stricken with grief, as he was their eldest son and a hard working boy. Sitting besides the coffin, Mrs Liseli hugs the little boy, Sepiso, close to her, while she mourns Likando.

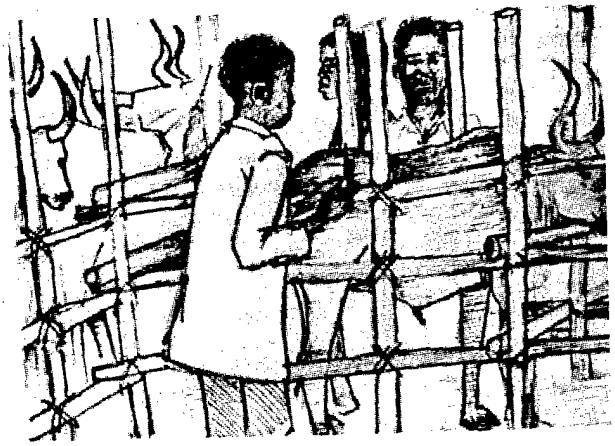
In the weeks following the death of Mr Liseli's ox, more cattle have died. The Veterinary Assistant has come to visit the farmers. He has explained that anthrax can spread quickly from one animal to the next when carcasses are opened and the

soil gets infested with spores. He tells the farmers they should bury animals that have died of anthrax. Some farmers refuse to do so. They say they want to eat the meat, and some want to keep the hide to show it to the owner of the animal.

Also Mr Liseli has lost another of his oxen. It was one of the oxen that pulled the sledge on which the meat of the first dead ox was carried to the village. But Mr Liseli does not doubt the words of the Veterinary Assistant. He thinks of what happened to Likando and decides to bury the animal. The Veterinary Assistant tells him that he should dig a very deep pit, so that dogs and hyenas cannot reach the dead animal. Mr Mundia, a friend of Mr Liseli has come to help him.



The Veterinary Assistant has explained to the farmers that anthrax spores can stay in the soil for many years. For this reason it is dangerous for animals to graze in an area where anthrax has been before. Once an animal is infected with anthrax, it will almost surely die. The disease spreads very fast in the body so that treatment nearly always comes too late. To prevent cattle from getting anthrax they have to be vaccinated every year before the time that the grass is so short that they will also swallow soil while grazing.



The following year, at the beginning of the cold season, when the grass is still long, the Veterinary Assistant visits the farmers again, to vaccinate their cattle. Mr Liseli has brought all his cattle to the crushpen, and he has asked all other farmers to do the same. Only a few farmers refuse while many are willing to vaccinate. The Veterinary Assistant is pleased that most farmers are cooperative and the farmers are pleased that their cattle are now being protected from anthrax. But most happy of all is Mr Liseli himself, knowing that his animals and his family are safe from this terrible disease.

That same year anthrax came to the village again. Luckily, Mr Liseli's cattle and the cattle of most other farmers, had been vaccinated in time, and all of them survived. Unfortunately some animals still died of anthrax. These belonged to farmers who had not allowed their cattle to be vaccinated. One of these farmers, Mr Sinaneli even lost all his oxen. Mr Liseli offered to lend Mr Sinaneli oxen, so that he could plough his field in time. Mr Sinaneli gratefully accepted the offer. When he came to Mr Liseli's kraal to collect the oxen, he said to him: *'Now that I have known anthrax myself I can see that I have been stupid. I should have listened to you before. Next year I shall make sure that my cattle are vaccinated in time, because I cannot afford to lose more animals to this disease!'*

Anthrax has been recorded in the districts of Lukulu, Mongu, Senanga, Kalabo and Sesheke. Annual vaccination campaigns are held in these districts between June and September. For more information, contact your local Veterinary Assistant or Community Livestock Worker.

M. E. HUGH-JONES

D/WHO Collaborating Center, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA

The data given on the attached pages is the maximum vaccination use quoted by OIE during the past three years. A number of facets are obvious from these data:

(1) Large scale vaccination programmes are the rule more than the exception.

(2) In some countries, the production of vaccine is far in excess of any reasonable zoonotic need, given there is a structured, objective, national control programme.

(3) Other countries, e.g., Portugal, are persisting with vaccination programmes but in the apparent absence of any reported anthrax incidence over many years.

With such national capacity in hand, those tempted to use *Bacillus anthracis* as a biological weapon need only make minor modifications to switch from vaccine to weapon-fill production. Though it is advantageous to have an efficient aerosol delivery system and other modifications in order to have an effective battlefield biological weapon, some would go ahead with simpler systems if only at the terrorist level of attack.

Therefore, it is argued that structured and efficient anthrax control programmes will significantly reduce the need for vaccine production in the medium term. For some countries, an immediate halt to anything but limited tactical vaccination on farms with outbreaks is long overdue. Any efficient control programme for livestock is in reality be an eradication programme; in the present state of knowledge, this cannot apply to wildlife-anthrax until cheaper and more effective vaccine delivery systems can be developed. Internationally accepted standards for anthrax control are possible and long overdue; the present WHO manual[1] is quite adequate to supply this need.

If FAO and OIE were to support making "unexplained sudden death" a notifiable disease especially of ruminants, thereby initiating their compulsory veterinary examination, this would reveal the true extent of this zoonotic disease and the need for vaccination programmes. It is necessary to turn anthrax from being a legally notifiable livestock disease but passively regarded problem into one under proactive effective control.

Similarly, the annual reporting of numbers of animals vaccinated against anthrax (or vaccine doses/units produced - whichever is most convenient) to OIE should be made obligatory. Then, as general vaccination needs decrease and are replaced by tactical, individual farm orientated programmes, any country maintaining a high vaccine production would stand out and require international explanation.

Reference

1. Turnbull PCB, Böhm R, Chizyuka HGB, Fujikura T, Hugh-Jones ME, Melling J. Guidelines for the surveillance and control of anthrax in humans and animals. *WHO/Zoon./* 93.170.

Anthrax vaccine production in relation to livestock population

Data is irratically released and total vaccine production seldom reported. Therefore this listing does the best it can with the available data. In general the vaccine volume listed is the maximum during 1992-94 in order to demonstrate capacity, not control efficiency. In general dose-levels are decreasing. For the most recent 93/94 vaccination levels refer to the full global report.

Country	Total doses x10³	Cattle			Ovi-Cap			Others		
		yr	doses	pop x10³	yr	doses	pop x10³	yr	doses	pop x10³
Africa										
Algeria	-	92	1,982	1,392	na	-	-	na	-	-
Angola	-	92	818,038	3,100	na	-	-	na	-	-
Botswana	-	94	1,257,972	2,844	na	-	-	na	-	-
Burkino Faso	-	92	10,413	4,218	na	-	-	na	-	-
Burundi	-	92	11,900	432	na	-	-	na	-	-
Cameroon	-	92	300,000	4,700	na	-	-	na	-	-
Chad	214	93	90,648	4,400	93	4,510	4,906	94	348	15 pigs
Cote d'Ivoire	-	92	1,060	1,144	na	-	-	na	-	-
Eritrea	73	na	-	-	na	-	-	na	-	-
Ethiopia	-	93	162,639	27,000	93	1,250	41,000	93	4,010	7,000 eq
Ghana	-	93	128,244	1,195	94	4,064	4,633	na	-	-
Guinea	-	92	75,332	1,472	92	10,967	945	na	-	-
Kenya	-	93	406,090	11,000	na	-	-	na	-	-
Libya	-	94	3,000	215	94	97,000	6,754	na	-	-
Madagascar	-	94	2,103,842	10,287	94	1,741	1,950	na	-	-
Mali	-	92	96,174	4,996	94	21,221	13,750	94	1,264	-
Morocco	460	94	na	3,269	92	3,080,212	21,896	na	-	-
Namibia	-	94	1,553	2,234	na	-	-	na	-	-
Niger	-	94	46,768	15,140	na	-	-	na	-	-
Senegal	-	93	19,520	2,627	92	297	5,670	92	24	295 pigs
South Africa	-	92	770,525	10,506	93	25,793	28,600	na	-	-
Swaziland	-	93	1,661	608	na	-	-	na	-	-
Tanzania	-	92	238,082	13,220	92	9,651	12,666	na	-	-
Togo	-	92	44,500	280	94	60	3,100	na	-	-
Uganda	-	93	68,720	5,000	na	-	-	na	-	-
Zambia	-	93	317,110	2,984	na	-	-	na	-	-
Zimbabwe	-	92	220,000	5,376	na	-	-	na	-	-

Country	Total doses x10³	Cattle			Ovi-Cap			Others		
		yr	doses	pop x10³	yr	doses	pop x10³	yr	doses	pop x10³
North & Central America										
Canada	-	93	300	12,249	na	-	-	na	-	-
Haiti	-	94	33,719	1,450	94	14,027	1,001	na	-	-
South America										
Brazil	9,654	93	-	144,154	93	-	31,710	na	-	-
Chile	-	94	1,298,740	3,461	na	-	-	na	-	-
Ecuador	-	92	6,686	4,580	na	-	-	na	-	-
Peru	-	92	800,000	4,042	na	-	-	na	-	-
Uruguay	-	94	4,058,308	9,508	na	-	-	na	-	-
Venezuela	-	92	47,869	13,586	93	7,706	1,322	93	150	2,956pig
Asia										
Bhutan	-	93	785	241	na	-	-	na	-	-
Cambodia	-	94	90,000	2,257	na	-	-	na	-	-
China	-	93	985,000	78,635	na	-	-	na	-	-
Iran	-	94	2,500,000	6,697	94	52,000,000	69,429	na	-	-
Israel	-	92	4,939	342	92	2,518	350	na	-	-
Israel Cnt Tr	-	93	97	17	92	1,400	551	na	-	-
Jordan	-	94	11,360	76	94	118,760	3,824	na	-	-
Korea DPR	-	93	12,424	1,300	na	-	-	na	-	-
Korea Rep	-	93	540,171	2,814	na	-	-	na	-	-
Kuwait	-	92	3,608	7	92	1,515	35	na	-	-
Laos	-	92	11,000	842	na	-	-	na	-	-
Mongolia	-	92	1,300,000	2,693	92	1,607,000	19,224	na	-	-
Philippines	-	92	9,326	1,677	na	-	-	na	-	-
Syria	-	92	57,677	787	93	1,566,408	16,157	na	-	-
Thailand	-	92	58,728	5,669	na	-	-	92	115,406	4,694bf
Turkey	-	94	678,552	11,377	93	1,147,534	51,530	na	-	-
Europe										
Albania	-	92	431,984	633	92	3,129,739	2,791	92	1,982	220pig
Austria	-	93	1,050	2,336	93	45	380	94	41	3,816pig
Bulgaria	-	93	764,866	1,575	93	3,359,091	5,426	94	500,000	2,680pig
Estonia	-	92	71,222	601	92	1,041	137	na	-	-
Greece	-	93	12,061	624	62,288	13,994	93	520		996pig
Hungary	-	92	577	1,050	na	-	-	na	-	-
Italy	-	92	77,759	8,737	92	550,239	13,094	na	-	-
Latvia	-	94	1,600	1,031	94	100	114	94	1,000	4,875pig
Lithuania	-	92	90,531	2,196	94	343	605	94	133	1,360pig
Netherlands	-	93	40	4,797	na	-	-	na	-	-
Poland	-	94	20	7,000	na	-	-	na	-	-
Portugal	239	03	-	1,375	93	-	6,530	94	-	2,664pig
Romania	-	94	4,197,167	3,865	93	12,051,000	12,997	na	-	-
Slovakia	-	94	321	-	na	-	-	na	-	-
Spain	-	93	42,480	4,976	92	50,000	27,700	na	-	-
USSR	-	91	15.4mil	115,600	91	243.5mil	140,600	91	77.6mil	75,600pig
Kyrgyzstan	-	92	1.6mil	930	92	10.7mil	9,830	na	-	-
Moldova	-	93	1.3mil	824	93	1.3mil	1,466	93	55,318	1,074pig
Russia	-	93	53.3mil	-	93	41.6mil	-	93	3.9mil	-pigs
Tajikistan	-	93	1.0mil	1,162	93	0	2,843	93	14,320	42pigs
							93	41,194	40eq	
Ukraine	-	93	20.8mil	-	93	6.1mil	-	93	317,140	-pigs
Uzbekistan	-	94	3.4mil	5,300	93	5.1mil	10,109	na	-	-
Yugoslavia	-	92	118,063	1,975	92	130,178	3,095	92	616	3,844pig
Croatia	-	92	185,000	590	na	-	-	na	-	-
Macedonia	-	94	28,168	285	94	111,294	2,656	na	-	-
Serbia	-	94	55,000	-	na	-	-	na	-	-
Slovenia	-	94	4,218	504	94	113	504	na	-	-

Anthraxin - a skin test for early and retrospective diagnosis of anthrax and anthrax vaccination assessment

E. SHLYAKHOV

Infectious Diseases Unit, Sheba Medical Center, Tel-Aviv University, Tel-Hashomer 52621, Israel

To avoid errors in clinical diagnosis of acute cutaneous anthrax (98-99% of cases encountered now), bacteriological confirmation is always required. However, the isolation of the pathogen is only partially successful.

During the first days after the onset of the disease, a progressive dying off of *Bacillus anthracis* inside the skin lesion occurs; this happens as a result of the antagonistic activity of local ubiquitous flora together with an early use of antibiotics. Thus, the "bacteriological-positive" stage of the disease is distinctly time-limited and, usually, at the time of the development of the eschar, no further culture can be obtained[1].

In addition to this, the late appearance of anthrax antibodies (at least 10-15 days after the onset of the disease) means that serological methods (e.g. ELISA) are useless for early diagnosis. Therefore these tests are also of only partial value for retrospective diagnosis[2,3]. An alternative immunologic diagnostic test using "Anthraxin" has been developed.

Anthraxin[R] is an antigenic preparation, derived from an unencapsulated *B. anthracis* strain. Intradermal administration of Anthraxin elicits, after 18-24 hours, a local erythema and skin induration in acute or recovered anthrax patients. The response can still be observed 48 hours later. This reaction appears in vaccinated humans and animals as well.

Histological and immunological studies on Anthraxin allow one to recognize this skin reaction as a reaction of delayed hypersensitivity which mirrors the anthrax cell-mediated immunity. Anthraxin has been licensed in the former USSR since 1962 for use in medical practice as a test for early and retrospective diagnosis of human anthrax and for comparative assessment of different anthrax vaccines, including evaluation of different vaccination routes and dose efficacy[4].

In the present study the Anthraxin skin test (AST) was compared with *B. anthracis* isolation in the same patients. Of 984 acute anthrax patients tested the results were as follows. In the first 3 days of the disease, 90 out of 110 tested patients were anthraxin positive (81.8%) while 78 of 187 showed positive culture (41.7%). During days 4-7 of the disease, skin positivity was recorded in 249 of 278 patients (89.5%) versus 71 with positive culture of 222 patients tested (32%). Within days 8-14, 332 out of 354 patients yielded positive skin test (93.8%) and only 23 of 127 patients showed positive culture (18.1%). During the 3rd week of the disease there were 166 of 168 anthraxin-positive (98.8%) in comparison with 8 positive cultures of 75 tested patients (10.6%). During the 4th week of the disease, no culture was obtained from 14 patients while 72 or 74 patients demonstrated positive skin test (97.3%) (Fig. 1).

The age and the sex of patients had no influence on the AST rates, while early administration of penicillin combined with streptomycin somewhat lowered these rates by 4 to 5%. However, the appearance of anthrax lesions on the cheek, on the front of the neck and on the chin increased positive rates by 5 to 7%.

During the "bacteriological-negative" period, starting one month after the onset of the disease, 950 anthrax patients were tested with Anthraxin. The positivity rates were as follows. Within 29 to 44 days after the onset of acute anthrax, 65 of 66 cases (98.5%); within 45 days to 3 years, 337 of 363 cases (92.8%); within 4 to 15 years, in 377 of 455 tested persons (82.8%) and within 16 to 31 years after recovery from acute anthrax, in 48 of 66 persons (72.7%) (Fig. 2).

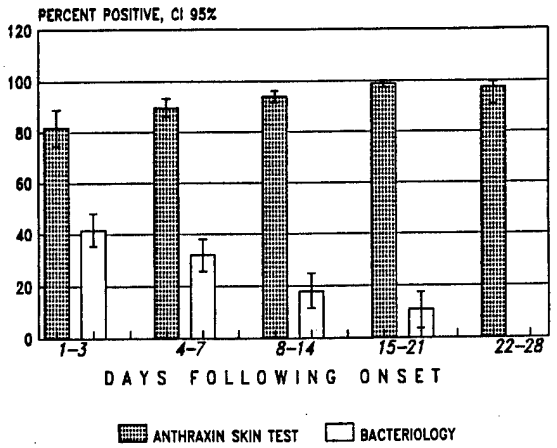


Figure 1. Anthraxin skin test diagnosis in patients with acute cutaneous anthrax versus *B. anthracis* isolation within the "bacteriological-positive" stage of the disease (per cent: positive skin reactions/pathogen isolation).

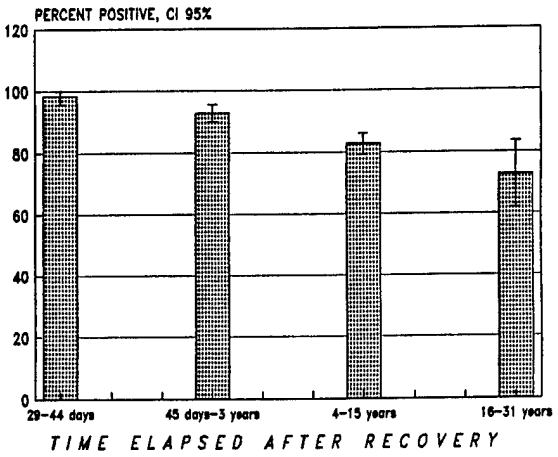


Figure 2. Anthraxin skin test retrospective diagnosis in patients suffering cutaneous anthrax in the past (from 45 days to 31 years after recovery) within the "bacteriological-negative" stage of the disease (per cent positive reactions).

To evaluate postvaccinal immunity, 2509 healthy adults not previously vaccinated against anthrax and with no a history of anthrax were vaccinated with the human live anthrax vaccine (HLAV) "STI". Within this group, 468 subjects were immunized by skin scarification (13-16 x10^7 spores/dose), 786 persons were injected subcutaneously (5-6 x10^7 spores/dose) and 1255 subjects were administered aerosolized HLAV in doses varying from 1.2 to 64 x10^7 spores.

AST were performed in separate groups of vaccinated subjects on days 2-5, 7, 15, 30, 90, 180 and 365 following vaccination, once for each person vaccinated during this study. The pattern of positive AST, regardless of route and dose of administered HLAV, appeared in all groups of vaccinated subjects as a similar, statistically significant five-phase curve. In the first (latent) phase, 2 for 5 days after vaccination, a slow increase of positive skin test rate (28.1% on an average) was observed. In the second (exponential) phase (on day 15) there were 61.3% on an average positive skin reactions. On day 30 (phase 3), an overall decline of Anthraxin positivity reaching,

on average, 40.2% was recorded, followed by a relative restoring of the skin positivity rate towards day 90, with an average of 53.1% (phase 4) (Fig 3). After this, a continuous gradual decrease of the Anthraxin positivity rate reaching, on average, 30% at the end of one year's observation was observed (phase 5).

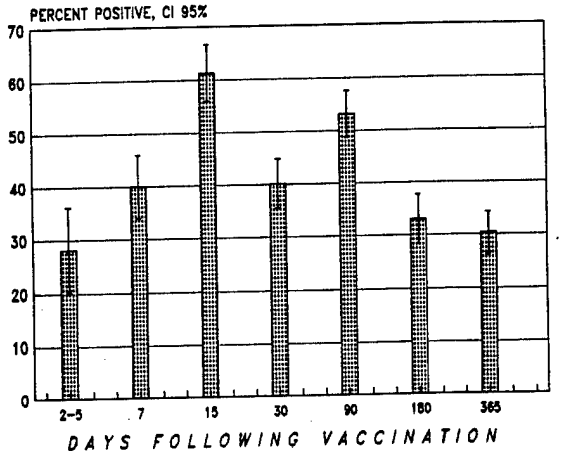


Figure 3. Anthraxin skin test positivity rates pattern in humans vaccinated with a live anthrax vaccine (during a one year survey).

The temporary decrease of positive AST on day 30 can probably be explained by the partial depletion of host macrophages due to the released lethal anthrax toxin by the multiplying vaccine organisms. This transient anergic state is followed by a relative restoring of AST positivity (toward day 90) induced by a new flow of previously primed macrophages.

Conclusions

The anthrax skin test seems to be a reliable and simple method for early and retrospective diagnosis of cutaneous anthrax. AST is 7 to 17 times more sensitive than the bacteriological method and can be used as an alternative test in cases which have not been confirmed by bacteriology or serology.

AST appears to be useful for comparative live anthrax vaccine testing and for conferred postvaccinal immunity assessment both in humans and animals. Anthraxin was recommended in the former USSR for the evaluation of the immunogenic potency of the reference live anthrax vaccine[5].

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Anthrax delayed hypersensitivity and the isolation of the vaccine strain

E. SHLYAKHOV

Infectious Diseases Unit, Sheba Medical Centre, Tel-Hashomer, Israel

The reluctance to introduce mass vaccination in the former USSR with human live anthrax vaccine STI was due to the fear of reversion of the vaccine strain to its virulent precursor. In order to dispel these doubts, one of the designers of the vaccine, N. N. Ginsburg, stated that the STI vaccine never produced harmful side effects, and that the vaccine strain disappears rapidly from the host's tissues[1,2].

This statement, however, was never confirmed by direct immunological observations.

In our study on this, five groups of guinea pigs, 6-10 animals per group, were vaccinated subcutaneously in the inguinal region with $4x10^7$ spores of STI vaccine. Two, 5, 7, 15, and 20 days following vaccination, separate groups of guinea pigs were tested intradermally with anthraxin. Specimens taken from the regional lymph node (RLN), liver, spleen, lungs, kidneys and heart blood were cultured at the same times.

The STI strain was isolated on day 2 from the RLN, spleen and lungs. On days 5 and 7 the strain was obtained from RLN, and spleen as well; on day 15, from the RLN only. On day 20 no vaccinal strain STI could be isolated any more.

In contrast, the anthraxin skin test demonstrated a continuous increase in positive reactions, from 1 of 6 (16.6%) on day 2 to 9 out of 10 guinea pigs (90%) on day 20.

Thus, the clearance of the vaccine strain and the induction of post-vaccine cell-mediated immunity showed a close inverse correlation (r= -0.92), suggesting that cell-mediated immunity, expressed by anthraxin skin reactions, is responsible for the disappearance of the vaccine STI strain from immunized host tissues.

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Protective efficacy of anthrax vaccine against parenteral challenge: a guinea pig model

SUPRODJO HARDJOUTOMO and M. BHAKTI POERWADIKARTA

Balai Penelitian Veteriner, Jalan R. E. Martadinata no.30, Bogor 16114, Republic of Indonesia

Summary

A group of anthrax vaccinated guinea pigs were used in this investigation; IgG antibodies to the vaccine was measured by ELISA. Vaccinated guinea pigs showed a variety of antibody titres; of these, 40% showed no elevation of their antibody titres at all. Challenge using virulent strains of *Bacillus anthracis* was conducted at 4 weeks after vaccination. It was concluded that there was a correlation between antibody ELISA titre and degree of protection against anthrax in anthrax vaccinated guinea pigs.

Introduction

Animal anthrax has been known to occur in Indonesia since 1885 and it is now endemic in certain areas of the country[2,3]. In those areas, preventive programmes against anthrax (namely annual mass vaccination in livestock) are undertaken routinely[2]. In ruminants, the ELISA technique may be used to monitor IgG antibody to vaccine[4]. The aim of the investigation was to determine whether there is correlation between ELISA titre and the degree of protection against anthrax in vaccinated animals.

Material and method

Immunisation

Local healthy guinea pigs weighing 300-400 g, were immunised as follows. There were altogether 6 groups of 12 guinea pigs, 5 vaccinated groups and 1 non-vaccinated group as the control. Sterne live spore vaccine made by PUSVETMA (Government Animal Biological Product Laboratory, Surabaya) was given as a single subcutaneus dose of 0.1 ml of 1.0×10^7/ml spore suspension. Blood samples were taken from all animals at 0 and 3 weeks later and IgG antibodies to vaccine measured by ELISA.

ELISA

The ELISA procedure was performed as described previously[4].

Parenteral challenge

Four weeks after vaccination, all guinea pigs were challenged by the parenteral route with 100 LD_{50} of *Bacillus anthracis* (virulent strains Bekasi and 17JB). The *B. anthracis* LD_{50} is 6.2×10^2 colony-forming units (CFU) for Bekasi strain and is $2.9 \times 10^{4.3}$ CFU for 17JB strain.

Statistical analysis

Data of ELISA and challenge test results were analysis statistically using X^2 test[1].

Results and discussion

The IgG antibody titres were expressed in ELISA unit (EU) with the highest and the lowest figures, 1024 EU and 16 EU, respectively. Based on their ELISA figures, vaccinated groups of guinea pigs showed a variety of responses, giving either low (44%), medium (2%) or high (13%) antibody titres when they were examined three weeks after vaccination. The untreated, non-vaccinated control group showed a variety of EU figures, in the order of 16 EU and 21 EU (data not shown).

As noted in Table 1, IgG antibody titres to vaccine showed a highly significant correlation (X^2=90,53; p<0,01) with degree of protection[1]. Antibody titres of vaccinated guinea pigs reflected their degree of protection against anthrax. The higher the antibody titres (100 EU - 200 EU), the higher the degree of protection. Vaccinated guinea pigs that had titres 200 EU and above had 100% protection whilst animals with titres less than 100 EU did not survive the challenge (Table 1).

It is interesting to note that in 40% of the vaccinated animals, there was no elevation of IgG antibodies to vaccine; both pre- and post vaccination ELISA titres, were 16 EU.

Table 1. Correlation between ELISA titre and degree of protection against anthrax in vaccinated guinea pigs

Pre-challenge Antibody Titre (EU)	Σ Alive	Σ Dead	Total	Degree of Protection (%)	
<100	0	73	73	0/73	(0%)
100-200	1	5	6	1/6	(16.7%)
200-300	4	0	4	4/4	(100%)
300-400	3	0	3	3/3	(100%)
400-500	2	0	2	2/2	(100%)
>500	8	0	8	8/8	(100%)
Total	18	78	96		

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Possible oral vaccination of wildlife against anthrax using *B. anthracis* strain Sterne $34F_2$ spores

F. GESSLER, J. RENGEL and H. BÖHNEL

Institute for Tropical Animal Health, Göttingen University, and Institute for Applied Biotechnology in the Tropics at the Göttingen University, Kellnerweg 6, D-37077 Göttingen, Germany

Our Institute has been engaged in developing and continually improving the IBT bioreactor technology for the production of veterinary vaccines in tropical countries. This technology is a continuous process where bacteria are grown in a fermenter under controlled optimal conditions, followed, by a cleaning and concentration step using hollow fiber filters[1].

For the production of *B. anthracis* Sterne spores, the process is divided into three steps:

i: vegetative multiplication in TYG medium
ii: washing of the bacteria with buffer solution and resuspension in sporulation solution containing Mg, Ca, Mn and Fe-ions.
iii: washing of the spores after complete sporulation, removal of buffer and final concentration of the spores in physiological saline.

Fig. 1 shows the diagrammatic set-up of our fermenter line. To avoid foam, but to maintain good aeration, the medium is pumped through a coil of silicone tube:, oxygen enters into the medium through diffusion, while volatile metabolites leave in the opposite direction. The yield under practical conditions in tropical countries is about 10^{10} spores/ml. The daily output is between 1 and 5 litres of culture, ie >1-5x10^{13} spores, with a fermenter vessel of maximum 20 litre capacity.

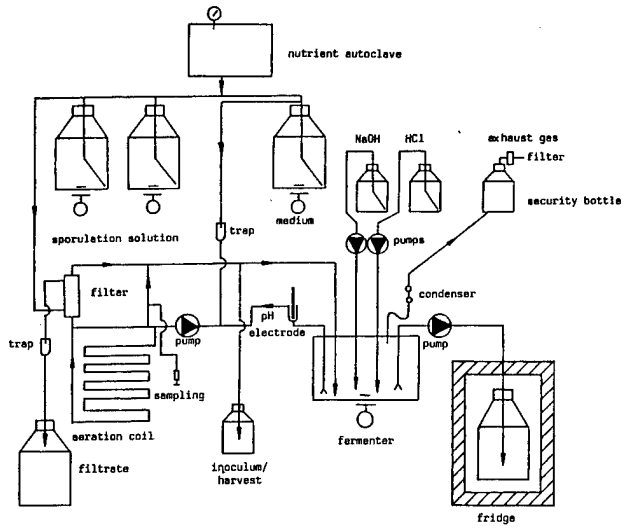

Fig. 1 Diagrammatic set-up of the anthrax spore fermenter

An outbreak of anthrax in the Luangwa Valley National Park in Zambia, affecting elephants, rhinos and ruminants, and endangering humans consuming parts of the carcasses, led to discussions with wildlife officers on possibilities of reducing the risk of this zoonosis in the highly populated game park. As the only practical means, the trial of an oral vaccination was taken into consideration; the parenteral application by darting was not considered to be financially viable[2]. As there were no scientific reports available, preliminary laboratory tests were planned to see whether this oral vaccination could be feasible.

More than 25 years since the first use, vaccination of wildlife in game parks is still done by shooting (bullets and darts) from the air, using aircrafts and special equipment[3].

The results of preliminary tests of oral vaccination are published elsewhere[5,6]. The genetic stability of several enteral passages of *B. anthracis* and *B. cereus* in guinea pigs is reported by Gessler *et al*[4].

Conclusions from the results of our laboratory tests are:

1 - it is possible to induce humoral immunity in guinea pigs by oral application of *B. anthracis* Sterne spores,
2 - the oral dose in guinea pigs is at least 10 times higher than the parenteral one
3 - vegetative forms of the bacilli are excreted with the faeces and are transformed almost immediately into spores.
4 - spores survive in dried elephant faeces for a long time (laboratory tests after one month; with droppings of guinea pigs after one year)
5 - parenterally vaccinated guinea pigs do not excrete viable bacteria in the faeces.

Oral vaccination with live spores should be tried now in several target animals kept in captivity to find out:

- necessary doses,
- possible seroconversion as a measure of penetration of spores into the host, and
- acceptance of different baits containing the spores.

It must be kept in mind that live vaccines, and especially those where immunogens are excreted by the vaccinee, are to be used only in endemic areas which are already contaminated with the corresponding pathogen. An accidental uptake of *B. anthracis* Sterne spores which had been excreted in faeces could lead either to a natural vaccination and/or a natural booster of already vaccinated animals conferring a protective immunity.

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Anthrax vaccine-induced seroconversion in zebra and elephant in the Etosha National Park, Namibia

P.M. LINDEQUE[1], C. BRAIN[2], W. VERSFELD[2] and P.C.B. TURNBULL[3]

[1]Ministry of Environment and Tourism, Private Bag 13306, Windhoek, Namibia.[2]Etosha Ecological Institute, P.O. Okaukuejo, via Outjo, Namibia. [3]CAMR, Porton Down, Salisbury, Wiltshire SP4 OJG, UK

Introduction

Considerable insights have been gained into various aspects of anthrax in Etosha National Park over the past thirteen years (Turnbull *et al.* 1989, Lindeque 1991, Lindeque & Turnbull 1994). Knowledge regarding the persistence of anthrax spores in the ground and water, the seasonal patterns of the disease, the spreading of the anthrax spores by scavengers and the distribution of spores as shed in the infected animals have elucidated many of the former unknowns about the disease in Etosha.

However, despite this considerable progress, there remains a great deal to learn about anthrax in Etosha. Because anthrax is potentially one of the most crucial factors affecting the development of an efficient management strategy in Etosha, it is imperative that conclusive tests be used to assess its effect on ungulate population dynamics. An integral part of this is understanding how the effect of the disease can be modified by vaccination.

Anthrax vaccination in livestock has been performed for over a century, but little is understood about the immunological response in the animal. The same vaccine as has been used in livestock for over 50 years is now commonly used in wildlife populations (De Vos *et al.* 1973). Antibody levels against anthrax protective antigen are not necessarily a good indicator of the level of protection against the disease (Ivins & Welkos 1988), but laboratory experiments showed that the live spore vaccine, although inducing lower anti-PA levels than the human vaccine, afforded greater protection (Turnbull *et al.* 1986, Ivins & Welkos 1988). It is thus reasonable to believe that animals with a measurable titre induced through vaccination with the Onderstepoort live spore vaccine will be more resistant to the disease than those with no titre.

This study, therefore, was aimed at measuring the titre over time, in order to work out an optimal vaccination schedule, ie, a schedule which will induce and maintain a high titre throughout the year.

The first wildlife species studied were zebra (*Equus burchelli*). A similar vaccination trial is underway for elephants in Etosha and the zebra trial was important in establishing a methodology for capture, vaccination, recapture and laboratory work.

Aim

To assess the vaccine-induced seroconversion against anthrax-specific protective antigen (PA) in zebra and elephant, using the Onderstepoort live spore vaccine.

Methods

Zebra

Nine female zebra, two of which had been vaccinated 11 months earlier, were immobilized and radio collared. Initial immobilizations occurred in the Okaukuejo-Leeubron area (within the anthrax enzootic area, see Figure 1). They were immobilized from a vehicle with a cocktail of 5 mg Etorphine hydrochloride and 5 mg Detomidine hydrochloride. The zebra were antidoted with Diprenorphine at 2.4 times the dose of the Etorphine. Palmer 2cc darts or Pneudart 1cc darts were fired from a Pneudart 171 rifle for the immobilizations. Immobilized animals were bled (femoral vein) and vaccinated with 2 ml anthrax live spore Onderstepoort vaccine. Animals were aged on initial immobilization. They were re-immobilized, bled and re-vaccinated/not vaccinated according to a predetermined protocol (Table 1). Two animals could not be located for the assessment of annual antibody titre, despite being radio collared.

We found that collared animals became extremely wary of the immobilizing vehicle after the third immobilization. This was undesirable as it was then only possible to dart the animals with marked disturbance of the animal and the herd and, if possible, future trials should have a maximum number of three immobilizations per animal.

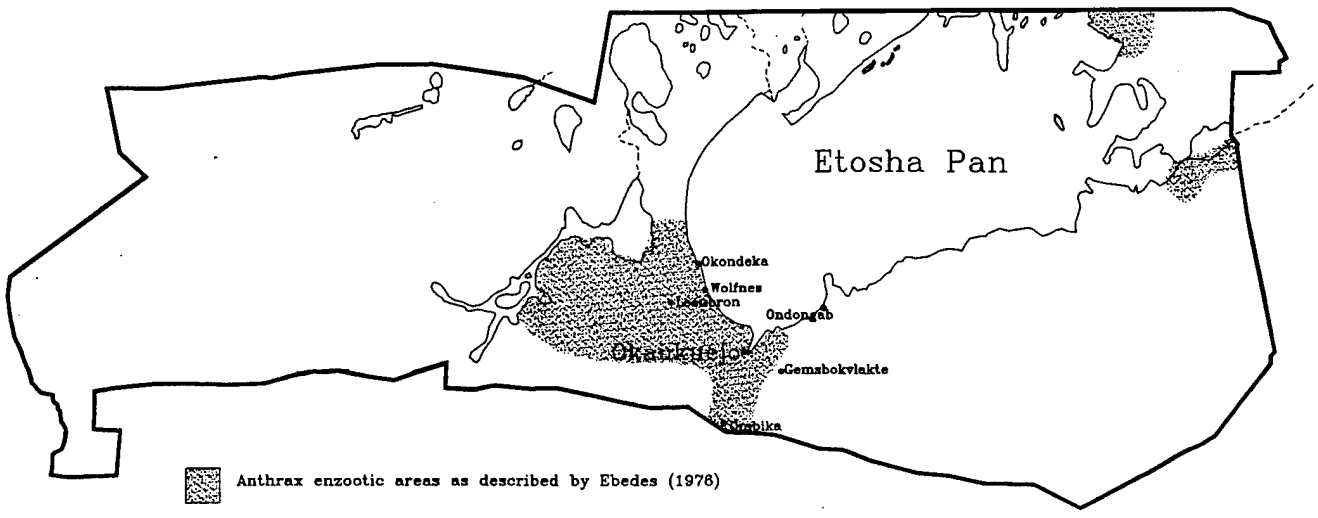

Figure 1. Map of Etosha National Park showing the anthrax enzootic areas described by Ebedes (1976).

Effectiveness of a vaccination schedule was determined by the appearance of a measurable anti-PA antibody titre, acknowledging that the presence of antibodies does not necessarily prove that the animals are totally protected against the disease. This study depended on the *in vitro* enzyme-linked immunoassay (ELISA) test for antibodies to anthrax PA as described by Turnbull *et al.* (1986). Long-term observation on mortality differences in vaccinated and unvaccinated groups may provide more data on the long-term correlation between antibody titre and actual protection levels.

Elephants

Ten adult elephant bulls were immobilized and radio collared. Initial immobilizations occurred in the Okaukuejo area during the dry season. Animals were immobilized from a vehicle with a cocktail of 18 mg Etorphine hydrochloride. The elephant were antidoted with 60 mg Diprenorphine. Immobilized animals were bled and vaccinated with 2 ml Anthrax Onderstepoort live spore vaccine. Animals were measured, sampled and radio-collared on initial immobilization. Five of the animals were re-immobilized, bled and re-vaccinated approximately 2 months after the initial vaccination (Table 2). The results from the sampling after 1 year were not yet available at the time of this report.

Table 1. Anti-PA titres in vaccinated zebras in Etosha National Park

Zebra no.	Date	Vaccine dose			Anti-PA titre (reciprocal of dilution)
		Dose no. no.	Volume (ml)	Months after first vaccination	
1*	27.02.93	1	2	0	weak (16)
	10.05.93	2	2	2.5	32
	07.07.93	3	2	4	256/512
	02.11.93	-		8	128/256
	NL **				
2*	27.02.93	1	2	0	32
	12.05.93	-		2.5	128
	02.11.93	-		8	negative
	10.04.94	2	2	13	negative
3	08.03.93	1	2	0	negative
	06.05.93	2	2	2	64/128
	07.07.93	3	2	4	256
	19.11.93	-		8	64
	09.04.94	4	2	13	32
4	08.03.93	1	2	0	negative
	05.05.93	2	2	2	weak (16)
	06.07.93	3	2	4	256
	02.11.93	-		8	256
	06.04.94	4	2	13	32
5	08.03.93	1	2	0	negative
	07.05.93	2	2	2	128
	05.07.93	3	2	4	128
	NL **				
6	08.03.93	1	2	0	negative
	12.05.93	-		2	weak (16)
	18.11.93	-		8	negative
	07.06.94	2	2	15	negative
7	09.03.93	1	2	0	negative
	12.05.93	-		2	64
	02.11.93	-		8	negative
	07.04.94	-		13	negative
8.	09.03.93	1	2	0	negative
	10.05.93	-		2	negative
	03.11.93	-		8	negative
	07.04.94	2	2	13	negative
9	09.03.93	1	2	0	negative
	07.05.93	2	2	2	negative
	Killed by lion				

* Vaccinated with 1 ml on 18.03.92

Results

Zebra

Anaesthetic induction in all the animals was smooth and there were no mortalities associated with the immobilizations. The results of the anti-PA titre in vaccinated zebra are shown in Table 1. Initial titres were measurable, but very low, only in the two previously vaccinated zebra. There was no evidence of naturally acquired immunity. Two to three months after a single dose, antibody titres were either low or undetectable and without a booster, antibody was undetectable by 8 months. One of the zebra was killed by a lion, but had shown no measurable titre after the first vaccination. A booster at 2-3 months resulted in a substantial titre.

In 4 animals that got a second booster (i.e. dose 3) at approximately 5 months, it was found the titre 4 months later was not higher than it had been after dose 2; it was either the same or reduced by one titration unit. This was interpreted as indicating that boosted antibody following dose 2 aborted the "mini-infection" of dose 3, consequently rendering dose 3 ineffectual. Animals that received dose 2 had considerably lower titres 13 months after dose 2 while animals that did not receive dose 2 had no measurable titre after a similar time period (including zebra number 2 which had essentially received two annual vaccinations).

Table 2. Anti-PA titres in vaccinated elephants in the Etosha National Park.

Elephant no.	Date	Vaccine dose			Anti-PA titre (reciprocal of dilution)
		Dose no. no.	Volume (ml)	Months after first vaccination	
1	15.08.94	1	2	0	negative
2	15.08.94	1	2	0	128
3	16.08.94	1	2	0	negative
	06.10.94	2	2	2	weak (8)
4	17.08.94	1	2	0	negative
	06.10.94	2	2	2	64
5	17.08.94	1	2	0	256
6	18.08.94	1	2	0	weak (8/16)
	06.10.94	2	2	2	128/256
7	18.08.94	1	2	0	negative
8	18.08.94	1	2	0	weak (16)
9	19.08.94	1	2	0	negative
	06.10.94	2	2	2	negative
10	19.08.94	1	2	0	negative
	06.10.94	2	2	2	negative

Elephant

In contrast to the zebra trial, two of the ten elephants sampled had definite antibody titres prior to vaccination, and two had evidence of weak titres (Table 2). It was unexpected to find naturally acquired immunity and this will probably make the interpretation of results more difficult. Of the five individuals given boosters at two months, two had developed no measurable titre from the first dose of vaccine, two had weak responses and one (which had a weak naturally acquired titre) had a stronger titre after the first vaccination. Due to financial constraints, the elephants that were re-vaccinated were those that were still in the Okaukuejo area at the required time. All individuals were due to be re-immobilised, sampled and vaccinated in August 1995 (one year after the initial vaccination). The serological results on these are not available yet.

Discussion & conclusions

Our results suggest that one booster is necessary at approximately 8 weeks after the initial dose to ensure development of a dependably measurable antibody titre in the first year of vaccination. A second booster (dose 3) within a few months of dose 2 is ineffective and probably can serve no useful purpose. It is difficult to conclude when subsequent boosters (after dose 2) are likely to be most advantageously administered. Clearly a sufficient time interval between doses 2 and 3 must be allowed. Our results suggest that an annual booster at least would be necessary after dose 2 to maintain a measurable titre although the effect on antibody titre after an annual booster will still need to be determined.

Unfortunately neither of the elephants with the naturally acquired antibodies were available for the 2-month booster, but the results of elephant number 6 (see Table 2) suggest that they probably have a high antibody titre after the first vaccination.

In this study, no attempt was made to measure the level of protection of the animals against anthrax. This was partly due to the difficulty of determining what a realistic challenge would be. In a study in the Kruger National Park, five out of six immunised impala died after an oral challenge with 10^7 spores (De Vos 1989), suggesting that protection is only relative to the level of exposure. It has been reported, however, that a vaccination campaign during an anthrax outbreak stops the outbreak within one to two weeks (Clark 1938, Sterne 1959, G.J. Scheepers, Phalaborwa Animal Hospital, Phalaborwa, RSA, pers. comm.). Given our results, this means that, even without a measurable titre, the vaccinated animals are protected from, natural infection. Furthermore, findings have shown that the live spore vaccines offer better protection than the human vaccine, although inducing lower anti-PA antibody levels (Turnbull *et al.* 1986, Ivins & Welkos 1988). All these observations seem to support the theory that both humoral and cell-mediated immunity are responsible for protection against anthrax.

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Remote mass vaccination of large free-ranging wild animals for anthrax using Sterne spore vaccine

V. DE VOS[1] and G. J. SCHEEPERS[2]

[1]National Parks Board, Skukuza, Kruger National Park, South Africa. [2]Phalaborwa Animal Hospital, Phalaborwa, South Africa.

Summary

An aerial method of immunizing free-ranging roan antelope in the KNP against anthrax was developed. This method involves the incorporation of the vaccine into a disposable dart-like projectile syringe fitted with a hypodermic needle. The syringe is fired from a specially made gun, and a helicopter is used to bring the operator within effective firing range of the animal. This method was improved by the use of a ballistic implantation method. It consists of a gun-like device capable of accurately delivering, over short distances, lightweight biodegradable implant projectiles (bio-bullets) containing the vaccine in pelletized freeze-dried form.

The Sterne spore vaccine was used. Although Sterne vaccine is, for all practical purposes, non-pathogenic in domestic animals, the situation in wildlife was unknown. The safety for wildlife was subsequently tested on 14 species of wild animals from the KNP. No adverse reaction to the vaccine or implants were detected.

The efficacy of the vaccine coupled to the method of application, was tested on guinea pigs, domestic goats *(Capra hirca)* and impala *(Aepyceros melampus)*. It was decided that immunity induced by these methods were sufficient to protect wild animals from anthrax as encountered in the field.

The method was subsequently field tested on 11 various species with good results and judged feasible for practical use. The relative ease with which the procedure was performed differed between the different species, the pachyderms being considered the easiest, while fast moving animals such as tsessebe *(Damaliscus lunatus)* and blue wildebeest *(Connochaetes taurinus)* were judged the most difficult to vaccinate.

It was estimated that a helicopter, consisting of a pilot, an observer and a marksman can do about 500 - 1,000 animals per day.

This method has now been applied successfully over 26 years within the KNP, immunizing roan antelope in a high risk anthrax region and safeguarding rare and threatened species from the decimating effects of an anthrax epidemic.

It was also used with great success on adjoining game ranches, prophylactically immunizing highly valued animals, and to suppress epidemic outbreaks. Mortalities were found to stop within two weeks after vaccination.

An outbreak of anthrax which occurred in a smaller nature reserve in South Africa, the Vaalbos National Park, was effectively stopped by applying this method and the vaccinating of a high percentage of animals within the area, applying this method.

The bio-bullet system was also used on animals which have been captured and held in a boma. Animals are vaccinated from the side of the boma or when they move out through a gate or crush. A herd of 600 buffalo was done in this way.

It is concluded that the aerial method of remote vaccination, as described, has the potential or ability to either suppress, stop an outbreak completely, or safeguard identified individuals or species, depending on how widely it is used. It is envisaged that remote vaccination, as described, can also be used with success in the domestic animal situation, working in inhospitable terrain under difficult circumstances. The remote vaccination procedure is considered a major breakthrough in the field of wildlife disease control and therefore, wildlife conservation.

Introduction

Control measures against anthrax, incorporating compulsory prophylactic vaccination of cattle with Sterne spore vaccine, was so successful that anthrax has virtually been eradicated from domestic animals and man in South Africa. A similar decline has been experienced in many countries throughout the world (Hugh-Jones, 1990).

Because of practical difficulties in applying these control measures to free-ranging wildlife, anthrax however, remains a threat to large wildlife conservation areas (Choquette and Broughton, 1981). Major epidemics have been, and periodically still are, recorded in areas such as Ruwenzori National Park in Uganda, Selous Nature Reserve in Tanzania, Etosha National Park in Namibia, Luangwa Valley in Zambia, and Kalahari Gemsbok, Vaalbos and Kruger national parks in South Africa (Choquette and Broughton, 1981; De Vos, 1990; De Vos and Lambrechts, 1971; Ebedes, 1977; Gainer, 1987; Pienaar, 1967; Turnbull, 1986, Turnbull *et al*, 1991).

Anthrax occurs endemically in the northern most corner of the Kruger National Park (KNP), but apart from this, four major anthrax outbreaks have occurred over the last 36 years, accounting for at least 12,000 deaths in various wild animals. A rare and endangered species such as roan antelope *(Hippotragus equinus)* has nearly been exterminated in the process. During the 1990/91 anthrax epidemic in the KNP, wildlife in adjoining nature reserves/game farms were also affected. Being commercial ventures, some wild animals on game farms have a considerable economic value attached to them. The loss of a single animal can be a serious financial setback to the owner, and warrants protection when threatened by a potentially decimating disease such as anthrax, even at great cost.

Earlier literature conveyed the belief that mass parenteral treatment and immunization of large populations of free-ranging wildlife were largely unpractical (Choquette and Broughton, 1981; Plowright, 1982). Choquette and Broughton (1981), however, demonstrated that with herd animals which can be corralled such as bison *(Bison bison)*, vaccination can be a feasible procedure. Long-stemmed hand-held automatic vaccination syringes were used from the sides of a crush. African animals which lend themselves to manipulation into corrals are herd animals which tend to maintain group-formation, inter alia Burchell's zebra *(Equus burchelli)*, blue wildebeest *(Connochaetes taurinus)* and African buffalo *(Syncerus caffer)*. Their wild and unruly nature, however, makes working from the side of a crush system with a hand held syringe very hazardous, if not impossible.

Harthoorn and Lock 1960 succeeded in vaccinating a very limited number of free-living African buffalo against rinderpest, by first immobilizing them with drugs delivered by darts and then injecting them with the vaccine. They estimated that a team of four people could immobilize and vaccinate about 20 to 30 buffalo daily. This method was therefore very time consuming and costly and had very limited application

under free ranging conditions.
With no solution forthcoming, a research programme was initiated with the object of finding practical and effective means to protect free-ranging large wild animals from the ravages of an epizootic such as anthrax.

A remote aerial vaccination procedure was subsequently developed, using disposable projectile syringes (De Vos *et al*, 1973), a ballistic implant (bio-bullet) (Johnson *et al*, 1984) and Stern spore vaccine. This method was tested for safety, efficacy and for practical feasibility during field trials and actual anthrax outbreaks in the KNP, Vaalbos National Park and private nature reserves/game farms bordering the KNP.

Materials and methods

Study areas

The KNP is situated in the north eastern corner of South Africa, between 22° 19' to 25° 32' latitude south and 31° 01' to 32° 02' longitude east. It borders on Mozambique in the east and Zimbabwe in the north. Towards the west and south human habitations, farmland and private nature reserves borders the KNP. It is a two million ha subtropical savanna woodland. It is completely fenced in and contains ± 3,700 kg biomass of mammals per 100 ha, made up of 149 different species of which 20 species can be classified as "large mammals", totalling about 140,000 individuals.

Along the western border of the KNP a series of private nature reserves and game farms exist, comprising about 300,000 ha. These areas are mostly managed as businesses and have a commercial value attached to most of their animals. Due to their proximity, similarity as regards animal populations, and free to-and-fro movements, major anthrax epidemics which occur in the KNP, invariably also affect these areas.

Vaalbos National Park is situated in the northern Cape Province near Kimberley, and consists of 18 120 ha of flat undulating plains. It is a semi-arid area with predominantly Kalahari thornveld type vegetation. It is inhabitated by 11 large animals species, including the endangered black rhino and the Addo version of the African buffalo.

*Projectile syringe system**

As described by De Vos *et al* (1973) a plastic disposable projectile syringe, with a smooth (barbless) needle shaft and pressurized by a spring mechanism, was used. The dart capacity allowed for a volume of 1.25 ml.

A single shot powder charged gun or a double barrelled air pressure powered dart gun was used to propel the projectile syringes. This resulted in intramuscular deposition of the vaccine.

Although this system as described is manufactured by the National Parks Board at Skukuza, any standard game immobilisation equipment can be used.

*Ballistic implant system***

The ballistic implantation, or bio-bullet system consists of a small biodegradable bullet, made of dehydroxypropyl cellulose (Klucel), and is designed to contain vaccines or pharmaceuticals in a freeze-dried state. The bio-bullets are sterile and individually sealed in doses of fifteen per clip. The clips are loaded directly into an air-powered gun and the bio-bullets are fed into the barrel by an under-barrel pump-like sliding action. The standard bio-bullet, as used in the KNP, is of 0.25 calibre, 12.7 mm long, 6.35 mm in diameter and has a payload (pellet) of 100 mg.

After implantation the action of body fluids serves to dissolve the dehydroxypropyl cellulose and to reconstitute the active ingredients contained in the pellet. Inoculants are now carried through the system, and within 10 hours the bullet has completely dissolved.

The whole process of pelletization, loading and sealing of clips under sterile conditions, has been automated, and has an output of thousands per day. A hand-operated pill-press and bullet-loader has however been developed in order to produce smaller numbers of bullets when used under experimental conditions. The hand system was used during this project and has an output of about 500 ready rounds per day.

Vaccine

A live virulent non-encapsulated *B. anthracis* vaccine strain, the so-called Sterne spore vaccine (Sterne, 1937; Sterne *et al*, 1942), being the major agent for the control of anthrax throughout the world and South Africa, was the vaccine of choice for the trials. This vaccine is produced by the Onderstepoort Veterinary Institute*** and conforms to the standards as set by the British Veterinary Codex.
The standard vaccine of 10 million spores per ml were used in the projectile syringes. Filled to capacity (1.25 ml) the syringe took 1.25 x 10^7 spores. For the bio-bullets the standard unencapsulated Sterne strain was used, but freeze dried before adjuvants were added. The freeze dried product was pelletized and forced into the "bullet". This also provided a dosage of 1.25 x 10^7 million spores.

Safety

Safety of the vaccine was tested on animals under controlled conditions. These were all animals that were kept for various reasons in captivity in the KNP. They were under surveillance and any untoward effects were noted.

Fourteen species were involved in these tests, viz. 50 elephant *(Loxodonta africana)*, 10 white rhino *(Ceratotherium simum)*, 10 black rhino *(Diceros bicornis)*, five giraffe *(Giraffa camelopardalis)*, 20 African buffalo, five kudu *(Tragelaphus strepsiceros)*, five waterbuck *(Kobus ellipsiprymnus)*, 30 roan antelope, 30 Lichtenstein's hartebeest *(Alcelaphus lichtensteinii)*, 12 blue wildebeest, 40 Burchell's zebra 30 impala *(Aepyceros melampus)*, 15 warthog *(Phacochoerus aethiopicus)* and 25 chacma baboon *(Papio ursinus)*. Both methods of application were employed and doses ranging from single up to five times the standard dose of vaccine were used.

Efficacy

The efficacy of the vaccine coupled to the method of application, was tested on guinea pigs, domestic goats *(Capra hirca)* and impala. These animals were vaccinated and after three weeks challenged with live anthrax spores.

Ten guinea pigs were implanted surgically with the bio-bullet and challenged after three weeks using the standard procedure with a laboratory strain of anthrax (17JB) at a dose of 200 MLD. This strain and dosage is routinely used for the screening of new batches of anthrax vaccine at the Onderstepoort Veterinary Institute.

Ten domestic goats were implanted with bio-bullets, using the standard remote procedure, and challenged after three weeks with a live virulent kudu strain at a rate of 10^3 spores parenterally. The kudu strain, which is a known highly virulent strain, was used after finding out that the laboratory 17JB anthrax strain was not virulent for goats.

Twenty impala were vaccinated, 10 with implants and 10 using the projectile syringe principle. They were challenged with 10^3 anthrax spores parenterally from the same kudu isolate as used for the goats. After losing 20% to anthrax after the first challenge, the challenge dose was decreased to 500 anthrax spores (kudu isolate).

* Manufactured by the National Parks Board, Skukuza.
** BallistiVet Inc., White Bear Lake, MN 55110, USA.
*** Onderstepoort Veterinary Institute, Onderstepoort, RSA.

Implantation success

The bio-bullet system was tested for implantation success on domestic goats, impala, eland *(Taurotragus oryx)*, kudu, roan antelope, blue wildebeest, waterbuck, Burchell's zebra, African buffalo, giraffe, and African elephant. The implants, or bullets, were filled with a placebo substance, consisting of lactate mixed with 1% magnesium stearate.

The animals received a known number of implants either from the ground, in the case of domestic goats and impala, or from the air (helicopter). These were then either chemically immobilized or slaughtered (by rifle shot) shortly afterwards, and the effects of implantation assessed. The testing and slaughtering of elephant and buffalo were done in conjunction with the culling programme in the KNP (De Vos *et al* 1983). In all cases the entry point of the bullet was located and probed for depth. In the slaughtered cases the bullet track was followed and the bullet located. Depth of implantation was measured and tissue damage assessed.

In all cases the actual number of shots that were fired as well as the number of shots that the marksmen scored as hits before checking, were recorded and compared to the actual amount of hits and penetrations that were achieved. Animals held under captivity were checked for obvious signs of abscessation afterwards.

Field application

The remote vaccination procedure that was developed made use of a helicopter (Bell Jet Ranger, four seater) with the rear doors removed. As depicted in Figure 1, one or two marksmen (shootists) were positioned in open rear doorways, loosely fastened to the helicopter with standard seat belts and specially made "monkey chains". These provided the necessary manoeuvrability and the ability to lean out of the open doorways without the danger of falling out. The herd or animals that had to be vaccinated were usually approached from one side, which invariably provoked an explosive flight reaction to the opposite side with the herd maintaining a semblance of group formation only until a difference in pace of individuals created a stringing-out of the group. The helicopter then moved in and out, working from the back to the front. With big herds small groups were cut out and taken aside. The helicopter positioned for shots at about five to six metres from the ground and about six to 12 metres from the animal to be vaccinated. The helicopter travelled at roughly the same speed and direction as the animal when the shot was delivered.

The practical feasibility of the method was first tested on various species in the KNP. A vaccination campaign was simulated on a representative field of large animals, their reactions observed and the ease of vaccination assessed. The remote vaccination method was also used to prophylactically immunize a roan antelope population within an anthrax danger area annually over a 23 years period. It was also used to safeguard individual animals in the KNP during a major epidemic, and to control outbreaks of anthrax in the Vaalbos National Park and private game farms bordering on the KNP.

Results and discussion

Safety

Although the Sterne vaccine has been found, for all practical purposes, to be non-pathogenic for domestic animals (Sterne *et al*, 1942), the same was not known about wild animals. The objective therefore was to test it in a representative sample of the spectrum of animals to be vaccinated. The same basically applied to the dehydroxypropyl cellulose, the substance that the ballistic implants (bio-bullets) are made of. Abscessation was another possibility which had to be considered.

No untoward effects were detected in the initial 14 wild animal species and 282 individual animals, which were included in the trial and were inoculated with anthrax vaccine under controlled conditions, utilizing the projectile syringe and bio-bullet systems. The vaccinated animals were kept under observation for weeks, and in some cases months afterwards. McCulloch and Achard (1965) also reported no harmful effect after the vaccination of three giraffe, nine wildebeest, three topi *(Damaliscus korrigum)* and four eland with anthrax, haemorrhagic septicaemia and blackquarter vaccines.

On the strength of these findings it was decided that the Sterne spore vaccine was safe for use in vaccination campaigns on wild animals. The validity of this assumption was borne out by subsequent applications. In no instance has there ever been any indication that the Sterne spore vaccine has actually caused the death of a wild animal.

Figure 1. Remote vaccination from a four seater Bell Jet Ranger helecopter, with marksman positioned in open rear doorway

Efficacy

The Sterne vaccine as used, remains to this day, essentially as originally formulated, the major agent for the control of anthrax throughout the world. As described by Sterne (1939) 300,000 and 600,000 to 1200,000 spores per dose, which were used for sheep and cattle respectively, elicited an immune response which was retained for at least a year. Both the projectile syringe and bio-bullet allows for 1.25 x 10^7 spores per dose, which is about 10 times more than is needed for cattle and must theoretically be considered quite sufficient for immunization purposes of wild animals. The largest land animals, rhino and elephant, weigh respectively five and 10 times more than cattle. The validity of this reasoning was further borne out by trials that were run on guinea pigs, goats and impala.

The guinea pigs that were implanted, were challenged in the usual manner in which commercial Sterne spore vaccines are screened. All animals survived, indicating that the implants provided the necessary protection.

Of the vaccinated goats and impala challenged with 10^3 spores of the kudu strain, six out of 30, or 20%, died from anthrax. This is slightly better than a control group which was vaccinated with the standard vaccine in the standard way, where three out of 10, or 33% died. This created the impression that the kudu strain might be too virulent and that the challenge dose could be too high. The challenge dose was subsequently lowered to 500 spores and the test repeated on 10 impala, which survived. The control group, which received no vaccine, formed part of an experiment where the objective is to find the lowest number of spores from the kudu strain that will consistently cause mortality in impala, if given parenterally. These experiments are at this stage at a point of titration where 100 spores consistently cause death in impala. The chances are therefore good that the challenge dose of 10^3 spores, as used for the vaccine tests, were too high and could have overloaded the immune responses of the animals. This was also experienced in previous vaccine trials in the KNP when 83% of vaccinated impala died from a challenge dose of 1 x 10^7 spores of the kudu strain *per os*. The survivors were found to be carriers of the virulent form of anthrax (De Vos, 1990). It is assumed that immunization breakdown might even happen in the field, especially during the height of an outbreak when infective doses are high.

All considered, it was decided that the efficacy of the remote vaccination methods, was good enough to protect animals against anthrax under field conditions. The validity of this assumption was later borne out by field application when anthrax outbreaks on game farms and a small nature reserve were stopped within three weeks after vaccination *(vide infra)*.

Implantation success

The projectile syringe system, having been successfully in operation for many years for the immobilization of wild animals, needed no further testing. The bio-bullet has never been used on African game before and had to be tested for implantation success.

Depending on the angle of the shot, either intramuscular, subcutaneous or intradermal depositions, or ricochets were achieved in domestic goat, impala, eland, kudu, roan antelope, blue wildebeest, waterbuck and Burchell's zebra. At relatively right angles effective penetration of 1.5 to 5 cm into the muscular tissue was invariably achieved. This was usually associated with little bleeding from the wound opening and minute trauma along the bullet track. In one case in an impala, a large blood vessel was nicked and a haematoma developed. A few impala and domestic goats also showed immediate but transient lameness after receiving the implant. One impala suffered temporary concussion from a direct hit on the neck vertebrae.

When shooting at an acute angle the bio-bullet either lodged intradermally or ricocheted. The same was experienced previously with the dart system. It was however, noted with relief that experienced marksmen were confident of judging hits, ricochets and misses. In tests, by either killing or chemically immobilizing animals that had been vaccinated (implanted) and counting the number of hits, ricochets and misses, it was found that this confidence was not misplaced. The number of hits recorded by the marksmen during flight invariably agreed very closely (more than 95%) with the actual hit/miss/ricochet rates as recorded during tests.

With the standard 0.25 calibre system, penetration of the skin was not achieved in buffalo and giraffe. Straight angle hits invariably bounced back towards the operator. By adding 200 mg ballast to the standard bio-bullet the hide of the buffalo was penetrated but still no success was achieved with the giraffe. The hides of these animals are not only thick, but seem to be very tough, elastic and fibrous and throw a bio-bullet back in a trampoline-like fashion.

As was expected, no skin penetrations were recorded in elephant bulls. In the case of female and immature elephant however, effective penetration occurred in nearly 50% of straight angle shots with the standard 0.25 calibre bio-bullet. All other bullets either lodged in the skin or ricocheted when an acute angle was used. No "bouncers", as in the case of buffalo and giraffe, were recorded. This surprising and somewhat anomalous success rate in an animal which is considered to have the thickest hide of all land mammals, is ascribed to the fact that elephant skin is less elastic and more brittle than that of buffalo and giraffe.

As indicated by the results, the standard 0.25 calibre implantation system causes mild injury in small animals such as domestic goats and impala. It however, seems to be ideally suited to the medium category species represented in this study by eland, kudu, roan antelope, blue wildebeest and zebra. From these, extrapolations can be made to other species.

Field application

The procedures as described under "Materials and methods" were tested with great success on various species in the KNP. Minor adjustments sometimes had to be made to fit peculiarities of different species.

The elephant population is widespread in the KNP. They are highly social and form family groups which vary in size from four to 20. Bulls are either found alone or form transient associations with family groups or other bulls. Being large, they are easily spotted from the air, are relatively cumbersome in movement and run relatively straight when chased by a helicopter. Family groups also tend to stay together and individuals are recognizable. It was found that the helicopter could easily keep up with a group and vaccinate virtually as fast as the rifle could be manipulated. The penetration efficiency of the bio-bullet is too low at this stage and the projectile syringe is used.

The giraffe population is also widely distributed throughout the KNP, though densities vary considerably with high concentrations occurring in the central areas. They tend to avoid dense tree and shrub thickets. They are semi-gregarious and are encountered singly (mostly old bulls) or in small groups of two to six animals. Occasionally loose associations of 30 to 40 giraffe are seen. They are also easily spotted from the air. As can be expected from their ungainly appearance they have virtually no way of evading the helicopter and darting can be done easily. The giraffe is therefore a very good candidate for a vaccination programme. The only drawback is that no penetration as yet could be achieved with the ballistic implantation system. Disposable projectile syringes have to be used at this stage.

Buffalo are quite numerous and are widely distributed throughout the KNP. They are highly gregarious and associate in herds of up to 1,000 individuals and more. The average herd size in the KNP is 300 animals and is easily spotted from the air. Bulls separated from the breeding herds prefer thickets or reed beds in the vicinity of perennial water. When disturbed by a helicopter they run relatively straight and it is quite possible to maintain herd formation by using sheep dog tactics with the helicopter. It was however found that by forcing a herd from behind, a difference in the individual pace of the buffalo causes "string" formation, with the fast young animals in front and the slower calves and older animals making up the rear. Sometimes the herds splits up into a few "strings". It was found relatively easy for the helicopter to move along the "string" and vaccinate the animals from rear to front. Buffalo, under these circumstances run relatively straight and are not difficult to dart from the air. Using this method, it was calculated that 60-70% of a herd consisting of 300 to 400 animals could be vaccinated in less than 30 minutes from contact to finish. With the herds it was also possible to repeatedly separate smaller workable units from the main herd. Due to the toughness of the skin, projectile syringes have to be used.

The low density eland population is widely scattered in the northern half of the KNP and they occupy a wide range of savanna habitats. They are gregarious and loose herds of up to 60 may be encountered. They favour parkland savanna habitats near perennial water and are therefore more easily spotted than kudu. They also demonstrated very little in the way of evasive tactics when chased by helicopter. Once spotted, implanting was easily executed. Due to their low density strip-search is however, indicated. Bio-bullets can be used.

Burchell's zebra is the second most numerous large mammal in the KNP and occur very widely, with the exception of dense shrub and tree thickets or dense riparian vegetation. Their highest densities occur on open plains, where they characteristically occur in association with wildebeest. Zebra are highly gregarious animals and under optimal conditions on open short-grass plains from loose aggregations numbering several hundred, or thousands. When disturbed from the air they present a straight and uncomplicated running pattern and were easily implanted. It was also possible to cut out small groups consecutively. Bio-bullets should be used.

Roan antelope is considered a very rare and endangered species in the KNP. They are found mostly on the northern basaltic grassland-savanna plains in small groups ranging from four to 16. Their colour blends in with the background and they are extremely difficult to track down and strip search is indicated. When chased by helicopter roan antelope do not run straight and crosses over frequently, which makes for a very elusive target. Bio-bullets are indicated.

The tsessebe (*Damaliscus lunatus*) is a very scarce species, with the highest density occurring on the basalt plains along the eastern half of the KNP. They have a distinct predilection for open, lightly wooded savanna plains and are semi-gregarious, occurring in herds numbering from four to 10. They are therefore relatively easy to track down by air, although strip search is still indicated in those areas. When chased by a helicopter, tsessebe presented a very devious running pattern. Bio-bullets should be used.

Blue wildebeest are numerous and are widely distributed throughout the KNP, although their densities vary considerably. They favour open short-grass plain or lightly wooded open savanna habitats. They are highly gregarious animals and under optimal conditions form loose concentrations numbering several hundred. The high density areas account for more than 80% of the wildebeest population and they are easily spotted from the air. The blue wildebeest in full flight from the helicopter characteristically toss its head from side to side with the body reacting in an exaggerated weaving run, which provides a very elusive target. It was possible to cut out consecutive small groups to be vaccinated. Of all animals tested, blue wildebeest are considered the most difficult to vaccinate but are still within the realms of feasibility. Bio-bullets are used.

Judging from these trials, the field operations phase of the ballistic implantation procedure must be judged feasible. The relative ease with which the procedure was performed however, varied with the different species, elephant being considered the easiest with giraffe, buffalo, eland, zebra, waterbuck, kudu, roan antelope, tsessebe, and blue wildebeest in order of increasing difficulty.

The sample of 11 species tested is considered large enough to be representative of the large mammal populations of Africa. It also allows extrapolations to be made to other species and other circumstances.

Animals such as impala and warthog are considered too small to vaccinate from the air. With these, and animals of similar size, vaccination from the ground will have to be contemplated. This will conceivably be a slow procedure, but will at least ensure the survival of viable breeding nuclei in the face of devastating disease.

The inability to mark every animal that was vaccinated was not considered a disqualification, even in herd animals such as buffalo, blue wildebeest and zebra. By working systematically this problem was circumvented to a great extent and the operators were confident of a high (more than 60%) implantation success rate in big herds. Work with mathematical models of epidemics suggests that in no case must the proportion of immune animals approach 100% before spreads halts (Fox *et al*, 1970). In the case of measles 70 to 80% immune has been cited (Fox *et al*, 1970). Information from a major anthrax outbreak in the KNP suggests that further spread of anthrax is dependent on a certain density of host animals. It was found that about 58,5% of the susceptible host population must be eliminated in order to reach a clinical endpoint of an anthrax epidemic (De Vos *et al*, 1995). This can be achieved by vaccinating the same percentage of animals.

Depending on the density of animals and by working systematically to implant all large animals in sight in the KNP, it was estimated that one helicopter with an experienced pilot, observer and marksman will be able to vaccinate about 500-1,000 animals per day. This depends a great deal on the amount of time spent on reconnaissance and search flights to be done.

The roan antelope population in the KNP has already been successfully vaccinated by this method for 23 years in what was considered, a high risk anthrax region in the northern part of the KNP. They were vaccinated annually, using projectile syringes until 1984, and the bio-bullet system since then. Being a low density species, locating the animals formed an essential and major part of the operation. For this purpose most of the searching was done by a light fixed wing aircraft, which reduced helicopter flying time, which is the most costly part of the operation. Further assistance was also rendered by an aeroplane during the ensuing darting (shooting) operations, by keeping the unvaccinated animals in sight.

During the last major anthrax epidemic in the KNP, rare and threatened species such as black and white rhinoceros, roan antelope, sable antelope, eland, tsessebe and Lichtenstein's hartebeest were located and immunized. This probably saved the already fast dwindling roan antelope population from becoming extinct during the 1990/91 anthrax epidemic.

During 1992 an anthrax epidemic occurred in Vaalbos National Park. The vaccination team moved in and vaccinated most of the rhino and buffalo and a high percentage of the

giraffe and kudu populations. The outbreak terminated within three weeks after vaccination.

During the 1990/91 major anthrax epidemic in the KNP, anthrax broke out on adjoining farms. This outbreak expanded and involved nearly all the private game farms adjacent to the KNP, covering a total of 200,000 ha. Vaccination was initiated and a total of 1,500 animals were vaccinated from a helicopter, using projective syringes. Mortalities again stopped within three weeks after vaccination.

The work on the private game ranches was performed by veterinarians in private practise, showing that the method of remote vaccination can be performed commercially and that there is a need for it in private enterprise. Although the cost is high, with the helicopter making up the major part of it, the value of some of the wild animals overshadows it completely.

Remote vaccination can also be performed on animals that can be caught en masse in a funnel and plastic lined corral system. Vaccination is performed from the side of the corral or from the side of the crush or gate as they pass through. A herd of 600 buffalo was successfully vaccinated in this manner.
It is also envisaged that this method, especially the bio-bullet system, can be used with success in the domestic animal situation, working in inhospitable terrain under difficult circumstances. In many of these areas no holding pens or crushes exist.

Conclusion

Enough evidence exist to warrant the conclusion that the remote vaccination method, using Sterne spore vaccine, is a feasible procedure for the control of anthrax in large free-ranging wild animals.

The method was used successfully as an annual prophylactic measure against anthrax in a roan antelope population from the KNP, and during a major epidemic, safeguarding scarce or rare species against the ravages of anthrax. It has also been calculated that by vaccinating 55 to 60% of the host population, an anthrax epidemic can effectively be stopped. It was also used on a smaller nature reserve and game ranches to effectively stops an outbreak of anthrax. For these reasons the remote vaccination procedure must be considered a major breakthrough in the field of wildlife disease control and wildlife conservation.

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The development of a dry, combined anthrax vaccine and the evaluation of its efficacy in experiments with laboratory and agricultural animals

*T. I. ANISIMOVA, T. V. PIMENOV, V. V. KOZHUKHOV, A. S. ARTEMIYEV, G. M. SERGEEVA, V. V. SEROGLASOV, N. V. SADOVOY, I. D. KRAVETS, G. D. ELAGIN, A. V. MASLOV, Y. A. YUDNIKOV, S. V. SURKOV and A. N. SHEVTSOV

*Laboratory of Particularly Dangerous Infections, State Institute for Standardization and Control of Biomedical preparations by name L. A. Tarsevich, Universitetskaya Street 46, 410071 Saratov, Russia *(authors unable to attend the workshop)*

In Russia during the 1980s, Sadovoy, N. V. with his colleagues developed a liquid combined anthrax vaccine, which possessed considerable advantages compared with live, and chemical vaccines. With its use, the period of development of immunity is shortened, the need for repeated applications is eliminated and its immunological efficacy is increased.

The problem of the best storage method for the combined anthrax vaccine, containing a spore suspension of the strain STI-1 and a protective antigen needed to be addressed. The aim of this investigation was the development a dry form of the vaccine for improvement of its operational properties, namely: increased stability during storage and transportation. Studies were directed towards the optimisation of freezing-drying regimes, to the choice a medium for drying and to storage conditions. Our experiments showed that selected conditions made it possible to obtain a dry preparation of vaccine, which maintained its immunogenic and biological properties under different temperature and time conditions: storage for 3 years at 4-6°C with short-term exposure at higher temperatures in a predetermined range.

A comparative evaluation of the immunological properties of the dry preparation of the combined and live vaccines, prepared using strains STI-1 and 55/5, was made. In the first stage the biological characteristics of these vaccines were studied in laboratory animals. After vaccination of guinea pigs, a higher level of immunity was noted in guinea pigs inoculated with the combined vaccine at a dose of 11-10^6 spores of strain 55/5 together with 40 ID_{50} of protective antigen (PA) and at a dose of 50-10^6 spores of strain STI-1 together with 40 ID_{50} PA.

The efficiency of the combined vaccine considerably exceeded that of the live monovaccine of strains STI-1 and 55/5. By physico-chemical, biological and immunological parameters this dry preparation conformed to requirements, demanded by the Pharmacopaeia Article for a liquid combined vaccine. Sheep vaccination was conducted in doses, based on those used for guinea pigs. A more expressed immune response and higher protective properties were noted in animals on day 10 after immunisation with the combined vaccine. Vaccination of calves at the age of 3-6 months was carried out with the combined vaccine at doses of 25x10^6 spores of strain STI-1 and 55/5 with 40 ID_{50} PA and with live dry vaccines of strain STI-1 and 55/5 at doses of 25x10^6 spores. In comparison with a control group, differences were not observed in body-temperatures, blood chemistry, SRE, *etc.* On the 14, the antibody titer in these animals exceeded that of animals vaccinated more than twice with the spore monovaccine.

So these studies showed that the developed dry preparation of combined vaccine in terms of harmlessness and reactogenicity did not differ from live vaccine, but exceeded it in immunological efficacy.

Development of a method for preparation and maintenance of the anthrax strain STI-1 and test strain Zenkovsky

*T. I. ANISIMOVA, E. V. PIMENOV, V. V. KOZHUKHOV, G. M. SERGEEVA, S. V. SURKOV, V. V. SEROGLASOV, A. V. MASLOV, YU. I. STROCHKOV, V. V. ZUBOV, V. I. KLIMOV and A. N. SHEVTSOV

*Laboratory of Particularly Dangerous Infections, State Institute for Standardization and Control of Biomedical Preparations by name L. A. Tarasevich, Universitetskaya Street 46, 410071 Saratov, Russia *(authors unable to attend the workshop)*

Vaccine strain STI-1 has been used in Russia for the production of a live-anthrax vaccine, and for human immunisation during more than 50 years. It was stated earlier that, during repeated culture of reference strain STI-1, accumulation of nonimmunogenic variants occurred, and eventually affected the strain quality and immunological efficacy of anthrax vaccines. In the past, traditional approaches were used to restore original properties of strain STI-1 and test-strain Zenkovsky, namely, selection of clones by cultural-morphological properties. This did not restore their biological and immunogenic properties in stable state for long periods. So, development of a method for preparing and maintaining reference strain STI-1 and test-strain Zenkovsky, guaranteeing the stability of their main biological properties needed to be addressed. Towards this end, the cultures were passaged through guinea pigs and clones selected on the basis of toxin (for STI-1) and toxin-capsule formation (for strain Zenkovsky) on the media of special composition and for their control of their genotype by the polymerase chain reaction (PCR) of the full length coding

This method considerably improved the biological characteristics of strains STI-1, and Zenkovsky. Stock cultures of reference strain STI-1, in contradistinction to the results of 6 *in vitro* generations, did not contain atypical RO-forms. All clones analysed had the complete gene *pag* and their immunogenicities exceeded those of reference initial cultures, prepared in a traditional way. After passage through animals and selection of clones monitored by the PCR method, the quality of the test-culture Zenkovsky was considerably increased too.

Microbial cultures of reference strain STI-1, obtained by the above method, were used for some years in the production of live dry anthrax vaccine, the quality of which exceeded in some respects, particularly immunogenicity of vaccines, the standard reference strains. So, the proposed method allows improvement of cultural-morphological, biological and immunogenic properties of studied vaccine strains and increases the quality of the reference strain STI-1.

Efficacy of the UK human anthrax vaccine in guinea pigs against aerosolised spores of *Bacillus anthracis*

M.N. JONES[1], R.J. BEEDHAM[1], P.C.B. TURNBULL[1], R.B. FITZGEORGE[1] and R.J. MANCHEE[2]

[1]*Anthrax Section, Research Division, CAMR, Porton Down, Salisbury, Wiltshire, SP4 0JG*
[2]*Medical Countermeasures, CBDE, Porton Down, Salisbury, Wiltshire, SP4 0JQ*

Summary

In tests aimed at determining the factors affecting the ability of the UK human anthrax vaccine to protect against infection in guinea pigs by inhaled anthrax spores, it was found that survival was affected by the particular challenge strain, the vaccine batch, the level of protective antigen and the adjuvant used. The official potency test on the vaccine did not, however, appear to be a reliable guide to the likely efficacy of the vaccine in this model.

Introduction

The current UK human anthrax vaccine, as manufactured by CAMR for the Department of Health, dates from the 1950's [1]. It is an alum-precipitated cell free filtrate of cultures of the non-capsulating toxigenic strain of *Bacillus anthracis*, $34F_2$, isolated by Sterne[7].

The formulation, manufacturing and test protocols for this vaccine have remained essentially unaltered since the licence was issued in the 1950's. Hence the vaccine is poorly defined antigenically which has led to difficulty in precisely defining dose specifications for unusual challenge conditions.

Little data is available on the efficacy of the UK vaccine in humans, although there is concern that it might have limited efficacy in the face of large challenges[9].

Protection data on aerosol challenges carried out in the 1950s[5] showed that the vaccine could protect rhesus monkeys against airborne challenge with spores of the Vollum strain of *B. anthracis*. Subsequent aerosol challenge experiments with guinea pigs[2] led to the description of the Vollum strain as "vaccine sensitive", since the vaccine produced good protection against this strain but only limited protection against challenge with the Ames and New Hampshire strains.

Further work on aerosol infection conducted at CAMR in 1991 (unpublished data) confirmed that the current UK vaccine affords poor protection to guinea pigs challenged with an aerosol of spores of the Ames strain.

The study reported here re-examined the protection afforded by the vaccine against aerosol challenges with the Ames, New Hampshire and Vollum strains of *B. anthracis*.

Materials and methods

Guinea pigs

Female Dunkin-Hartley guinea pigs were used.

Spores

Spores of the Ames, New Hampshire and Vollum strains of *B. anthracis* were prepared for use as described elsewhere in this volume[6].

Immunisations

The first protection trial was aimed at assessing the variability of protection induced against aerosol challenge by four selected batches of the UK vaccine and the effect of a supplementary adjuvant on protection. The four vaccine batches, chosen on the basis of their varied performance in the official potency test, were Batch 1 (code 355) which had failed the potency test, Batch 2 (code 360) which had passed the potency test well, and Batches 3 (code 354) and 4 (code 358) which had just met the criteria for passing in the potency test. Batch 1 was tested alone and in combination with a second adjuvant, Ribi Tri-Mix (Sigma).

The second protection trial, done with just Batch 1, was concerned with the effect of supplementing the vaccine with additional purified protective antigen (PA), again monitoring the additional effect of including Ribi Tri-Mix.

The guinea pigs given the UK vaccine were immunised by intramuscular injections at 3-week intervals of one-tenth of a human dose of the vaccine (0.05 ml vaccine + 0.05 ml saline). They were challenged 2 weeks after the third dose. Where the Ribi adjuvant was included, each dose consisted of 0.05 ml vaccine + 0.2 ml of the adjuvant reconstituted in saline according to the manufacturer's instructions. When the vaccine was supplemented with purified PA in the second protection trial, the immunisations consisted of 1 volume of vaccine mixed with 1 volume of saline containing purified PA at a level such that each guinea pig received approximately 0.65 µg PA (PA1) or 3.5 µg (PA2). These levels were effectively equivalent to 0.25 to 0.5 of the PA in a human dose of the UK vaccine (PA1) or five times that level (PA2). These estimates are based on the results of ELISA determinations on solutions resulting from addition of one part 0.1 M citric acid (pH 3.5) to an equal part of the vaccine held at 4 °C until the alum had dissolved[8]. By this method, the vaccine appears to contain approximately 1.25 to 2.5 µg of PA per dose. Control animals received equivalent volumes of saline, or saline + Ribi adjuvant or saline containing the requisite concentration of PA.

Prior to challenge, blood was taken from 2 guinea pigs in each group for determination of anti-PA titres by a modification of the inhibition ELISA described elsewhere[10].

Infective challenge

The guinea pigs were exposed for 5 (first protection trial) and 7 (second protection trial) min periods to aerosols of the Ames, New Hampshire or Vollum spores using a Collison nebuliser containing 10^9 - 10^{10} spores/ml in distilled water, connected to an animal exposure tube via a Henderson type apparatus[3,4]. This resulted in retained lung doses of 10^4 to 10^6 spores. The animals were then monitored for a further two weeks.

Results

Protection trial 1

The survival rates among the vaccinated and control guinea pigs exposed to spores of the Ames and New Hampshire strains of *B. anthracis* are summarised in Table 1.

Fourteen days after challenge, the surviving guinea pigs were euthanased. At postmortem, *B. anthracis* was detected in the lungs of 35 (71%) of 49 animals which survived exposure to the Ames spores and of 79 (98%) of 82 which survived exposure to the New Hampshire spores.

Table 1. Deaths of guinea pigs following aerosol challenge with spores of virulent *B. anthracis*.

Vaccination groups.	B1	B1+R	B2	B3	B4	CON	CON + R
Potency test result	F	F	P/W	P/W	P/J		
Ames @ 10^{0}	5/9	1/5	3/10	8/10	9/10	5/5	2/2
Ames @ 10^{-1}	2/9	0/5	3/9	4/10	3/10	3/4	
New Hampshire @ 10^{0}	2/10	1/5	1/10	2/10	2/10	3/5	1/2
New Hampshire @ 10^{-1}	0/9	0/5	1/10	1/10	1/10	3/4	

B, batch; CON, Saline control; F, Failed; P/W; Passed well; P/J, Just passed

Protection trial 2.

The survival rates among the guinea pigs used in this trial are summarised in Table 2.

Table 2. Deaths of guinea pigs following aerosol challenge with spores of virulent *B. anthracis*.

Vaccination Group	B1	B1 +R	B1+ PA1	B1+ PA2	B1 + PA1 +R	PA1 +R	CON	CON +R
Ames @ 10^{0}	8/9	1/10	6/10	4/10	1/8	0/8	9/9	1/2
Ames @ 10^{-1}	1/9	0/8	1/9	4/10			7/8	
Vollum @ 10^{0}	3/10	0/10	0/10	0/10	0/10	0/10	8/9	2/2
Vollum @ 10^{-1}	1/10	0/10	0/10	1/10			8/9	

B, batch; R, Ribi; CON, Control (saline); PA1, 0.9 µg total PA; PA2, 5 µg total PA

The guinea pigs that survived were again euthanased and postmortemed 14 days after challenge. *B. anthracis* was isolated from the lungs of 65 (97 %) of 67 of those that had been exposed to the Ames strain spores and of 95 (98%) of 97 of those exposed to the Vollum strain.

Discussion

Linear regression analysis of the results in the first protection trial revealed no correlation in the first protection test between the official potency test results on the different batches and the ability to protect guinea pigs following substantial respiratory challenge with spores of virulent *B. anthracis*. Giving "fail", "just passing", and "passing well" values of 1, 2 and 3 respectively, linear regression analysis (5 observations, 3 degrees of freedom) revealed $R^2 = 0.008$ ($p>0.05$) with Ames at 10^{0}, 0.492 ($p>0.05$) with Ames at 10^{-1}, 0.643 ($p>0.05$) with New Hampshire at 10^{0} and 0.762 ($p>0.05$) with New Hampshire at 10^{-1}. Although batch 2, which had passed the potency test well, performed better against challenge with undiluted Ames spores than batches 3 and 4 which had just passed the potency test, so did batch 1 which had failed the potency test. There was little to distinguish the results with the different batches in the remainder of the challenge tests and the virulence of the New Hampshire strain in these tests was limited in any event, with only 3 of 5 unvaccinated controls succumbing following exposure to the maximum number of spores possible in 5 min.

Analysed by chi-squared tests on a comparison of each vaccine preparation with that of batch 1 in the second protection test, significant improvements in protection by the UK vaccine were afforded against the undiluted (10^{0}) spore preparations of both Ames and Vollum strains by the Ribi Tri-Mix and PA2 supplements ($p<0.005$), by PA1 in the case of the Vollum strain ($p<0.005$) and by PA1 with the Ames strain ($p<0.025$). PA1 with Tri-Mix also performed significantly better than batch 1 alone ($p<0.05$). This is in agreement with previous conclusions[2]. Analysed by 2x2 χ^2 tests with Yates' correction for numbers ≤ 10, protection against undiluted Vollum spores was significantly greater than against Ames spores ($p<0.01$ for the vaccine alone; $p<0.001$ for unsupplemented and supplemented vaccine taken together). Protection against Ames spores was significantly enhanced when the vaccine was combined with Tri-Mix ($p<0.01$).

Full analysis of the serolgical results has yet to be completed but a preliminary scan of the readouts indicates that anti-PA titres in the different groups are in line with those noted in previous studies involving similar immunisation schedules[10,11,12]. The large proportion of lungs still harbouring *B. anthracis* in the surviving guinea pigs two weeks after challenge indicates that vaccine-induced protection does not infer rapid clearance of the infecting organism. This parallels similar findings reported elsewhere [5,6].

In conclusion, factors affecting survival of guinea pigs exposed to aerosols of anthrax spores were the particular challenge strain, the vaccine batch, the level of PA and the adjuvant used. The official potency test on the vaccine did not, however, appear to be a reliable guide to the likely efficacy of the vaccine in this model.

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Efficacy of a standard human anthrax vaccine against *Bacillus anthracis* aerosol spore challenge in rhesus monkeys

B. E. IVINS*‡, P. F. FELLOWS*, M. L. M. PITT†, J. E. ESTEP†, S. L. WELKOS*, P. L. WORSHAM* and A. M. FRIEDLANDER*

Bacteriology Division and Applied Research Division†, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, 21702-5011, USA*

‡To whom correspondence should be addressed

In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, revised 1985).

The views, opinions and/or findings contained in this publication are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

Summary

The efficacy of a licensed human anthrax vaccine was tested in rhesus monkeys challenged by an aerosol of virulent *Bacillus anthracis* spores. Adult rhesus monkeys were injected intramuscularly at 0 and 2 weeks with 0.5 ml of vaccine or phosphate-buffered saline. At 8 weeks, 38 weeks or 100 weeks, the animals were challenged by *B. anthracis* aerosolized spores. All immunized animals survived challenge at either 8 weeks or 38 weeks, and seven of eight animals survived challenge at 100 weeks. All control animals died 3 to 5 days after challenge. Serum from immunized animals possessed demonstrable antibodies to protective antigen by ELISA.

Introduction

Bacillus anthracis is the causative agent of anthrax, a disease primarily of herbivores, but one which humans can acquire through contact with infected animals or animal products. The anthrax vaccine licensed for human use in the United States, MDPH (manufactured by the Michigan Department of Public Health, Lansing, Michigan, U.S.A.), consists of aluminum hydroxide-adsorbed supernatant material, principally protective antigen (PA), from fermentor cultures of a toxinogenic, nonencapsulated strain of *B. anthracis*, V770-NP1-R[12]. Several recent studies demonstrated the partial efficacy of MDPH in guinea pigs challenged parenterally with *B. anthracis* spores of the virulent Ames strain[7-10,13], and, in a field evaluation in humans, a vaccine similar to MDPH showed protection against anthrax[2]. Unfortunately, no study has been reported on the efficacy of the vaccine in nonhuman primates against an aerosol spore challenge. Thus, the research reported here was conducted to determine the short-term, mid-term, and long-term efficacy of MDPH against inhalation anthrax in rhesus monkeys.

Materials and methods

Animals

Adult male and female rhesus monkeys *(Macaca mulatta)*, weighing 4.4 to 16.8 kg, were immunized intramuscularly at 0 and 2 weeks with 0.5 ml, the standard human dose, of the MDPH human anthrax vaccine. Control animals were given 0.5 ml of phosphate-buffered saline (PBS). Animals were challenged by an aerosol of *B. anthracis* spores of the virulent Ames strain at either 8 weeks, 38 weeks, or 100 weeks. Survival for 3 months after challenge was noted, and moribund animals were euthanized. Weekly pre- and postchallenge bleeds were drawn on all animals, and the sera were assayed for antibodies to PA by enzyme-linked immunosorbent assay (ELISA) either by an indirect method using baculovirus-produced PA[5], in which mouse monoclonal antibody to PA was first bound to the ELISA plates, or by a direct method in which *B. anthracis*-produced PA was bound to the ELISA plates (C. Rossi, personal communication). Blood was cultured quantitatively for 10 days after challenge as described previously[4].

Spore challenge

The virulent Ames strain of *B. anthracis* was obtained from the U. S. Department of Agriculture, Ames, Iowa. It was grown in Leighton-Doi medium, and spores were harvested and washed in sterile, distilled water as previously described[9]. The spores were purified by centrifugation through 58% Renografin-76, washed again, then resuspended in 1% phenol and stored at 4°C.

For aerosol challenge, spores were suspended to a concentration of approximately 1.5 X 10^9 CFU/ml, then heat-shocked at 60°C for 45 min. Eight-ml aliquots of the spores were used for aerosol challenge with a three-jet Collison nebulizer as previously described[3,4,11]. The concentration of spores in the aerosol (sampled in water in an all-glass impinger) and the aerosol inhaled dose (expressed as LD_{50}) were also determined as previously described[3,4,11]. An aerosol inhaled dose of 5.5 X 10^4 spores of the *B. anthracis* Ames strain was previously determined to be 1 LD_{50} in rhesus monkeys (B. Ivins, unpublished observations).

Results

All 10 of the immunized monkeys challenged at 8 weeks survived a small-particle aerosol inhaled dose of spores (255 to 760 LD_{50}), whereas all five PBS controls died 3 to 5 days after challenge (Table 1). Similarly, at 38 weeks, all three monkeys survived an aerosol spore challenge of 161 to 247 LD_{50}. At 100 weeks, the final group of eight immunized and two control monkeys were aerosol challenged with 239 to 535 LD_{50} of spores. Seven of eight immunized monkeys survived. Of all the surviving immunized animals, only one had a demonstrable, transient bacteremia, which lasted from days 2 to 6 after challenge. The bacteremia never exceeded 200 CFU per ml on the days assayed. The two control monkeys died 4 days after challenge. Terminal bacteremias in control monkeys that died during the study ranged from 4.7 X 10^6 to 5.5 X 10^8 CFU per ml.

Mean anti-PA ELISA titers before and after challenge are presented in Table 2. Immunized animals exhibited a substantial increase in titer after the 2-week booster and also after challenge at 8 weeks. By 99 weeks, titers dropped to a barely detectable level, but 2 weeks after challenge at 100 weeks, they rose sharply to a geometric mean of 28,265.

Blood was drawn for clinical evaluation every other day after challenge for 10 days from the three monkeys challenged at 38 weeks. The white blood cell counts increased, whereas red blood cell counts, hematocrit, and hemoglobin decreased.

Other parameters such as fibrin degradation products, fibrinogen, activated partial thromboplastin time, prothrombin time, and platelets were not affected.

Discussion

A human anthrax vaccine must protect against all forms of anthrax, including inhalation anthrax, which, although rare, is usually fatal. The data in this study demonstrate that the MDPH vaccine is highly efficacious against inhalation anthrax in rhesus monkeys. The rhesus monkey is a useful model for inhalation anthrax in humans, although there is currently no known surrogate marker or in vitro correlate of immunity that allows direct comparison of immunity in humans to that in monkeys. Although the current vaccine regimen in humans calls for doses at 0, 2, and 4 weeks, 6 months, 12 months, 18 months, and then yearly thereafter, in this study only two doses of vaccine, at 0 and 2 weeks, were required to provide substantial protection for almost 2 years. Based on this study's data, the MDPH human anthrax vaccine confers substantial protection against inhalation anthrax, and the recommended immunization regimen may be able to be reduced with respect to the number of doses.

PA is a major component of MDPH, and previous efficacy studies[6,10] demonstrated that PA must be present in a non-living anthrax vaccine or produced in a live vaccine. Other components such as edema factor, lethal factor, and cell-surface antigens may be present in some lots of MDPH and might affect the vaccine's efficacy. MDPH contains as an adjuvant aluminum hydroxide (Alhydrogel), which is a good stimulator of humoral immunity, but not cell-mediated immunity[1]. The high level of efficacy of MDPH in rhesus monkeys suggests that humoral immunity is important in the specific resistance of rhesus monkeys to anthrax. In guinea pigs, however, intramuscular immunization with MDPH only partially protects against a challenge with anthrax spores[7,10,13].

These findings suggest that the importance of various, specific immune mechanisms against inhalation anthrax may vary in different animal species, or that the ability of the licensed human anthrax vaccine to stimulate cell-mediated immunity may be greater in some species than others.

Table 1. Protection of rhesus monkeys by MDPH from aerosol challenge by *B. anthracis* Ames spores

Time after first immunization[a]	LD_{50}	Survived/total (%)		Time to death in days (range)
8 weeks				
MDPH	255-760[b]	10/10[c]	(100)	-----
PBS	189-435[d]	0/5	(0)	3 - 5
38 weeks				
MDPH	161-247[e]	3/3[c]	(100)	-----
100 weeks				
MDPH	239-535[f]	7/8[g]	(88)	4
PBS	511-535[h]	0/2	(0	4

[a]Monkeys were immunized intramuscularly at 0 and 2 wk with 0.5 ml of MDPH human anthrax vaccine. [b]Mean LD_{50} = 437. [c]All surviving monkeys had negative bacterial cultures through 10 days after challenge. [d]Mean LD_{50} = 303. [e]Mean LD_{50} = 203. [f]Mean LD_{50} = 330.[g]One of the seven surviving monkeys had a positive bacterial culture (days 2-6) after challenge. [h]Mean LD_{50} = 523.

Table 2. Anti-PA ELISA titers of immunized monkeys

Time after first immunization	Geometric mean titers
0 weeks[a]	ND[b]
2 weeks[a] (before second immunization)	14
8 weeks[a] (before 8-wk challenge)	919
10 weeks[a] (2 weeks after challenge)	7,879
99 weeks[a] (1 week before 100-wk challenge[c])	200
102 weeks[d] (2 weeks after 100-wk challenge[c])	28,265

[a] ELISA performed by using indirect method
[b] ND = Not detectable.
[c] Not previously challenged at 8 weeks or 38 weeks.
[d] ELISA performed by using direct method. Titres obtained by the direct method gave values which were approximately 1.71-fold greater than those obtained by the indirect method.

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Antibiotic prophylaxis for inhalation anthrax

M.N. JONES[1], R.J. BEEDHAM[1], P.C.B. TURNBULL[1] and R.J. MANCHEE[2]

[1]Anthrax Section, Research Division, CAMR Porton Down, Salisbury, Wiltshire, SP4 0JG, UK. [2]Medical Countermeasures, CBDE, Porton Down, Salisbury, Wiltshire, SP4 0JQ, UK

Summary

Ciprofloxacin and doxycycline protected guinea pigs from infection following inhalation of retained doses of up to 10^6 spores of the Ames or Vollum strains of *Bacillus anthracis* so long as administration of the antibiotics was continued. After antibiotic administration had been stopped, a small proportion of deaths resulted, with the time at which these occurred being 10 to 19 days later in the doxycycline group than in the ciprofloxacin group. At the time of termination of the experiments (17 and 25 days after stopping administration of ciprofloxacin and doxycycline respectively), *B. anthracis* was isolated from a large proportion of the lungs of the survivors.

Introduction

Respiratory anthrax, although rare as a naturally occurring disease, has long been fatal unless treated in time. The first choice antibiotic for the treatment of anthrax has long been penicillin, although there have been occasional reports of penicillin resistant strains[1,2,8,10]. Treatment of experimental inhalation anthrax with penicillin has had a variety of outcomes [3,4,6].

In putative combat situations, where numerous individuals might be faced with large doses of aerosolised anthrax spores, probably penicillin resistant and possibly combined with other infectious agents, penicillin is impractical from the standpoint of repeated administration, potential hypersensitivity reactions and possible lack of effect against the other agents. Recent studies have focused on more practical modern alternatives, ciprofloxacin and doxycycline[5,7].

This paper examines the effects of an aerosol challenge with spores of virulent *B. anthracis* on guinea pigs prophylactically treated with ciprofloxacin and doxycycline.

Methods and materials

Guinea pigs

Female Dunkin-Hartley guinea pigs (500-600 g at time of challenge) were used.

Spores

Growth from purity plates of Vollum and Ames strains of *B. anthracis* was transferred to sporulation agar slopes in 25 ml bottles which were incubated for several days at 28 °C. The growth from each slope was washed off with 3 to 5 ml of sterile deionised water (SDW) which was transferred to a sterile bottle and held at 62.5 °C for 15 min followed by subculture to check its purity. The 3 to 5 ml suspension of heat-shocked spores were then used to inoculate sporulation agar in Roux bottles which were incubated at 28 °C for approximately 2 weeks. The degree of sporulation was checked under a phase contrast microscope for the presence of >95% phase bright spores and the growth on the agar in each Roux bottle was then washed off with 25 to 50 ml SDW. The spores were centrifuged and resuspended in 65% (v/v) isopropanol and held overnight in the refrigerator. They were then centrifuged and washed three times in SDW, cultured for purity and the satisfactory lots pooled and counted.

Antibiotics

The antibiotics chosen, ciprofloxacin (Ciproxin^T, Bayer) and doxycycline (Vibromycin-D^T, Pfizer), were administered subcutaneously at two dose levels, 5 and 10 mg/kg every 12 h for ciprofloxacin and 4 and 8 mg/kg every 24 h for doxycycline. (In humans, the antibiotics would be administered orally, but this was not feasible with guinea pigs). The antibiotic treatment regime commenced two days prior to aerosol challenge and continued for 21 days post challenge.

Challenge

Two groups of 53 guinea pigs were challenged for 7 min with aerosols of spores, gently sonicated to ensure maximum dispersion, of the Ames strain (Group 1) or Vollum strain (Group 2) to give retained lung doses of 10^4 - 10^6 spores. In each set, 20 guinea pigs were given ciprofloxacin, 10 at the high dose level and 10 at the low dose level, and 20 others received doxycycline, again 10 at the high dose level and 10 at the low dose level. The remaining 13 animals were controls.

Administration of the antibiotic ceased 21 days after challenge; the guinea pigs were then monitored for signs of illness.

Antibiotic level determinations

Two non-infected guinea pigs were injected subcutaneously with the high level dose of ciprofloxacin (10 mg/kg) and 2 with the high level dose of doxycycline (8 mg/kg) using the same schedule as used in the main experiment. After 3 days of antibiotic administration, the animals, together with their untreated controls, were killed at 2, 4, 6 and 12 h hours after the last dose. Blood, kidney, liver, spleen and lungs were removed, macerated, and 20 ml aliquots of the suspensions placed in 4 mm sample wells cut into plates of Muller-Hinton agar inoculated with a lawn of *B. globigii*. Zones of inhibition were looked for around the sample wells.

Results

All the control animals died of anthrax within 72 h and anthrax as the cause of death was confirmed by M'Fadyean stained blood smears.

None of the animals on either of the antibiotics died during the 21 days of antibiotic administration, but some ulceration occurred at the sites of repeated injection with ciprofloxacin. After cessation of antibiotic administration, 7 animals died, 3 from the ciprofloxacin group and 4 from the doxycycline group (Table 1).

Table 1. Deaths of 7 guinea pigs following cessation of antibiotic administration

Antibiotic	Number of days after stopping antibiotic administration	Dose level	Challenge strain
Ciprofloxacin (terminated 17th day after stopping antibiotic administration)	4	Low	*Ames*
	5	Low	*Ames*
	5	Low	*Ames*
Doxycycline (terminated 25th day after stopping antibiotic administration)	15	Low	*Vollum*
	18	Low	*Vollum*
	20	High	*Ames*
	23	Low	*Vollum*

The guinea pigs which died after antibiotic administration had been stopped were postmortemed and the lungs, spleen and liver removed for bacteriology. *B. anthracis* was found in high numbers in all samples.

The guinea pigs given ciprofloxacin were euthanased and postmortemed 17 days after the withdrawal of the antibiotic. *B. anthracis* was present in the lungs of 35 (95%) of the 37 animals. Counts in 4 sets of lungs from the group challenged with the Ames strain ranged from 14 to 240 (mean 81) cfu/g and counts in 4 sets of lungs from the group challenged with the Vollum strain ranged from 24 to 192 (mean 93) cfu/g. The two sets of lungs which were negative, even with enrichment in BHI broth containing 7 mg/ml polymyxin B at 37° C, came from the low dose group challenged with Ames (1 guinea pig) and the high dose group challenged with Ames (1 guinea pig).

The animals on doxycycline were euthanased and postmortemed 25 days after stopping antibiotic administration. *B. anthracis* was present in the lungs of 27 (77%) of the 35 guinea pigs. Counts in 4 sets of lungs from each of the Ames and Vollum groups ranged respectively from 48 to 144 (mean 80) cfu/g and 72 to 1393 (mean 573) cfu/g. The 8 which were still negative even after enrichment were from the low dose group challenged with Ames (1 guinea pig), the high dose group challenged with Ames (5 guinea pigs), the low dose group challenged with Vollum (1 guinea pig) and the high dose group challenged with Vollum (1 guinea pig).

Distinct "peak" and "trough" levels could not be detected in the antibiotic level evaluation tests (Table 2). The zones of inhibition were too small to permit quantitative assessment of levels in the positive organs and the only organ to exhibit zones of inhibition at all of the time periods was the kidney. Zones of inhibition were obtained at 6 h with the lung suspensions for both antibiotics, and the sera and liver for doxycycline.

Table 2. Zones of inhibition seen around the sample wells cut into lawns of *B. globigii*

Time in hours after administration of antibiotics	Sample tested for antibiotic levels									
	Sera		Lung		Liver		Spleen		Kidney	
	C	D	C	D	C	D	C	D	C	D
2	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	+/+
4	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	+/+
6	-/-	+/+	+/-	+/-	-/-	+/+	-/-	-/-	+/-	+/+
12	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	+/+

C, ciprofloxacin; D, doxycycline; -, no zone of inhibition; +, zone of inhibition; +/-, only one animal of the two had organs which exhibited zones of inhibition.

Discussion

The results showed that ciprofloxacin and doxycycline protected guinea pigs from inhalation anthrax when challenged with the maximum retained dose of 10^6 spores of either the Ames or Vollum strains of *B. anthracis* so long as administration of the antibiotics was continued. After antibiotic administration had been stopped, a small proportion of deaths occurred; the time at which these mortalities occurred appeared to be dependent on which of the antibiotics had been administered with deaths in the doxycyline group taking place 10 to 19 days later than those in the ciprofloxacin group.

Administration of the antibiotics did not eliminate *B. anthracis* from the respiratory tract; a large proportion of the lungs were still positive for *B. anthracis* at termination of the experiments with both antibiotics and both strains. It is not known why more of the animals did not die after the antibiotics had been stopped and the patterns of the deaths from the two challenge strains is unexplained (Table 1). Some immunity may have developed during the 21-day period of antibiotic administration following challenge, but anti-PA antidbody was not detected by ELISA on sera from 16 animals (two from each combination of high and low dose, ciprofloxacin and doxycycline, and Vollum and Ames groups) examined 17 or 25 days after administration of ciprofloxacin and doxycycline (respectively) had been stopped (38 and 46 days respectively after challenge).

It is also difficult to explain the different times of the deaths occurring in the two antibiotic groups; counts in the lungs of the animals exposed to the Ames strain were broadly the same in the two antibiotic groups while, in those exposed to the Vollum strain, lung levels were substantially higher in the doxycycline group.

Lung counts done both before and after heating at 62.5 °C for 15 min revealed no significant differences, thereby indicating that the organism was present only in the spore form. This is in agreement with the conclusions of Henderson *et al*[6] who considered that any germinated anthrax spores found in lungs after aerosol exposure had, in fact, germinated after postmortem. With this in mind, we were careful to transfer the lungs as soon as possible after death to ice baths.

The occurrence of deaths, confirmed as due to anthrax, after antibiotic administration had been stopped, together with the persistence of spores in the lungs, parallel the findings of others[5,6] and support the concept that, following exposure to aerosols known to have, or suspected of having, large numbers of anthrax spores, administration of antibiotics such as ciprofloxacin or doxycycline simultaneously with vaccine may have to be continued for 6 to 8 weeks while vaccine-induced immunity develops.

In the antibiotic level evaluation, the detection of the antibiotics in the lungs confirmed the ability of these antibiotics to reach the initial site of infection, the respiratory tract. However, it is possible that administration of the antibiotics as aerosols would result in greater lung antibiotic concentrations. It would be interesting to know if this would increase the rate of clearance of *B. anthracis* from the lungs of animals infected by the aerosol route.

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Protection against experimental anthrax infection using fragments of Protective Antigen

S. F. LITTLE , B. E. IVINS, P. F. FELLOWS and A. M. FRIEDLANDER

Bacteriology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21702-5011, USA

In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, revised 1985). The views, opinions, and/or findings contained in this publication are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

Effective immunization against infection with *Bacillus anthracis* requires the presence of protective antigen (PA) in a vaccine which may be either a chemical vaccine combining PA with an adjuvant or a live vaccine expressing PA[8]. To determine which domains of PA are important in inducing immunity, the proteolytic fragments PA_{47}, PA_{37}, and PA_{17} of PA were tested for their ability to protect guinea pigs against infection with *B. anthracis*. Limited proteolysis of PA by chymotrypsin results in a 37-kilodalton (kDa) amino-terminal fragment (PA_{37}) and a 47-kDa carboxyl-terminal fragment (PA_{47}), while limited proteolysis with both trypsin and chymotrypsin results in PA_{47}, 20-kDa (PA_{20}), and 17-kDa (PA_{17}) fragments[2,6], the latter two being the amino-terminal and the carboxyl-terminal fragment of PA_{37}, respectively. Fragments were purified by chromatography on MonoS (Pharmacia Biotech, Uppsala, Sweden) in the presence of 5 M urea after limited proteolysis using chymotrypsin or chymotrypsin and trypsin[5]. Urea present in the PA_{47} and PA_{37} fractions was decreased to <0.5 M by buffer exchange in 20 mM Hepes, 50 mM NaCl, pH 7.55 (Hepes buffer) by using a Centriprep 10 device (Amicon Inc., Beverly, Mass.). To remove fragments possessing the carboxy-terminus of PA in the PA_{17} preparation, the PA_{17} fraction was passed over a monoclonal antibody PA 2II 10G4-1-1[4,5] hydrazide column after buffer exchange to 10 mM sodium phosphate, 0.15 M NaCl, pH 7.3 (PBS) using a desalting column. Urea was added (2 M final concentration) to the unbound fraction which then was subjected to buffer exchange to 50 mM Hepes, 2 M urea, pH 7.5 buffer by using a Centriprep 10 device. Fractions were stored at -70 °C. In addition to native PA, PA denatured with urea (PA_{UREA}) served as controls. PA_{UREA} was prepared by diluting PA to 1 mg/ml in Hepes buffer containing 5 M urea. After incubation for 1 h at room temperature, buffer exchange was carried out as described above for PA_{47}. The relative purity of each fragment preparation was assessed by Coomassie blue stained gels.

Female Hartley guinea pigs (350-400 g; Charles River) were injected intramuscularly (i.m.) with a single 16 µg dose in 0.5 ml of the PA fragment emulsified in Ribi adjuvant (trehalose dimycolate, monophosphoryl Lipid A, and cell wall skeleton in oil and Tween-80; Ribi ImmunoChem Research, Hamilton, Mont.) and were challenged i.m. 6 weeks later with 10000 spores (100 LD_{50}) of the Ames isolate of *B. anthracis*. Animals were observed for 14 days. Sera, obtained 3 days before challenge, were evaluated for anti-PA antibody by ELISA. The ELISA was as described previously[3] except that the second antibody was horseradish peroxidase conjugated to goat anti-guinea pig IgG (Kirkegaard & Perry, Gaithersburg, Md.), the diluent was PBS containing 0.5% gelatin and 0.05% Tween-20, and all incubations were carried out at room temperature.

A single injection with the whole PA molecule provided 75-81% protection, whether or not it had been previously treated with urea (Table 1). PA_{37} gave 56% protection, while neither PA_{47} nor PA_{17} afforded any significant protection. PA_{47} and PA_{17} were not very antigenic after a single injection, demonstrated by the low anti-PA ELISA titers, compared to the other preparations. Our results demonstrate that one of the critical epitopes involved in protection against infection resides on the PA_{37} amino-terminal fragment. These findings are in agreement with a previous study by Tedikov and Dobritsa[7] who reported that the amino-terminal 50 kDa fragment portion of PA retained immunological and protective properties, although no details were given. Previous research also demonstrated that the PA_{20} amino-terminal fragment of PA was not protective, while PA_{63} was protective[1]. Together these results imply that the protective epitope of PA_{37} is part of PA_{17}. The lack of protection observed with PA_{17} and PA_{47} may be due to the minimal immune response generated with these fragments. This suggests that the critical protective epitope(s) on each fragment, which may include conformational epitopes, may have been disrupted during the purification procedures.

Table 1. Protection afforded guinea pigs immunized with a single injection of various PA antigen preparations

Antigen	Survival/Total	%	anti-PA ELISA Titer*
PA	12/16	75	3198
PA_{Urea}	13/16	81	5040
PA_{47}	1/16	6	217
PA_{37}	9/16	56	1337
PA_{17}	0/14	0	153
PBS	0/8	0	96

*Geometric mean reciprocal dilution at 0.2 optical density reading at 405 nm

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Comparison of the efficacy of purified protective antigen and MDPH to protect non-human primates from inhalation anthrax

M.L.M. PITT, B. E. IVINS, J.E. ESTEP, J. FARCHAUS and A.M. FRIEDLANDER

U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Frederick, MD, 21702-5011 USA

Inhalation anthrax, caused by infection with the microorganism *Bacillus anthracis*, has a high fatality rate. The current licensed human vaccine, Anthrax Vaccine Adsorbed (MDPH), available from the Michigan Department of Public Health, is composed primarily of the protective antigen (PA) component of anthrax toxin. This vaccine has been shown to be effective under field conditions[1]. However, a series of six 6 doses within 18 months (followed by yearly boosters) is required. In addition, the vaccine is fairly reactogenic and the concentration of protective antigen varies from lot to lot.

Both the need for multiple immunisations and high reactogenicity of the vaccine in humans has led to research into an alternative vaccine which could protect quickly, with a much lower local reaction rate. All effective vaccines against anthrax are based on the presence of PA, an 83,000 MW protein[2].

The rhesus monkey (*Macaca mulatta*) is considered to be the most appropriate model for human inhalation anthrax[3]. Anthrax in the rhesus monkey, induced by respiratory exposure to spores of a virulent strain of *B. anthracis*, is a rapidly fatal illness, death occurring between the 2nd and 7th days postexposure[4].

In this study, we compared the efficacy of MPDH with a purified recombinant PA combined with Alhydrogel. Ten monkeys were immunised with MPDH; ten received 50 μg PA + Alhydrogel (containing 0.725 mg of metallic aluminum per dose), and two controls received Alhydrogel PBS. The immunisations, 0.5 ml, were given intramuscularly on days 0, and 28. The animals were bled weekly and their serum IgM and IgG titres determined by ELISA. The animals were challenged by aerosol exposure to a lethal dose (899 LD_{50} ± 62) of anthrax spores (*B. anthracis*, Ames strain) three months after immunisation. The controls died on days 3 and 7 respectively. All the MDPH monkeys were fully protected compared to 9/10 of the PA + Alhydrogel group. The one monkey in the PA + Alhydrogel group that died developed low level bacteremia and hemorrhagic meningitis. Thus, there was no statistical difference between the protection afforded by recombinant PA plus Alhydrogel compared to MDPH in this animal model of inhalation anthrax.

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Protective antigen-based engineered vaccines

YOGENDRA SINGH[1], KURT R. KLIMPEL[2], PRABODHA K. SWAIN[1], SEEMA GOEL[1] and STEPHEN H. LEPPLA[2]

[1]Centre for Biochemical Technology, Mall Road, Delhi-110007, India, and [2]Laboratory of Microbial Ecology, NIDR, NIH, Bethesda, MD, 20892, USA

Bacillus anthracis is fully virulent only when it produces a three component exotoxin and a poly-D-glutamic acid capsule[2]. Previous studies indicated that immunization with protective antigen (PA), a protein component of the toxin, provides complete protection to guinea pigs against challenge with a virulent strain of *B. anthracis*[1]. The currently used human vaccine (MDPH-PA) contains PA as a major component adsorbed on aluminum hydroxide[1]. The human vaccine has been shown to be of reduced efficacy against certain strains of *B. anthracis*[1]. Immunization with MDPH-PA vaccine sometimes causes mild pain and other undesirable reaction which may be due to toxin components adsorbed to aluminum hydroxide from the bacterial culture supernatant. The major aim of this study was to understand the molecular mechanism of action of anthrax toxin and then apply that knowledge to develop a safe and economical vaccine for anthrax.

Previous studies showed that PA bound on target cells is activated by cleavage with furin to produce 20- and 63-kDa fragments. Cleavage at the same sequence, RKKR167, can be achieved *in vitro* by very low concentrations of trypsin. The 63-kDa fragment (PA63) is believed to form an oligomeric, membrane-inserted channel involved in translocating the lethal factor (LF) and edema factor components to the cytosol[3]. Proteolysis with chymotrypsin *in vitro* generated 37- and 47-kDa fragments[4]. Receptor-binding and LF-binding activities were retained in both the 63-kDa and 47-kDa proteins. However PA cleaved with chymotrypsin was biologically inactive[4]. The role of the protease-sensitive sites in the biological activity of the toxin was studied by making mutant PA proteins.

The PA gene was subcloned into an *E. coli-B. subtilis* shuttle vector. The mutant proteins expressed from this plasmid in *B. anthracis* were purified to near homogeneity by fast protein liquid chromatography. As expected, deletion of amino acid residues 163-168 (SRKKRS), required for cleavage with trypsin, made PA completely resistant to trypsin[6]. Similarly, PA deleted at residues 313-314 (FF) was completely resistant to chymotrypsin[5]. Mutation of either the trypsin or chymotrypsin sensitive sites did not interfere with the binding of PA to its cellular receptor. The PA mutant lacking the trypsin-sensitive site was not cleaved by cell surface proteases, and hence could not bind LF[6]. However, the PA mutant having the chymotrypsin-sensitive site deleted was cleaved by cell surface proteases and could bind LF. The trypsin and chymotrypsin site PA mutants were both non-toxic in combination with LF (Table 1). These results indicated that the trypsin site mutant was non-toxic because it is unable to bind LF. The

chymotrypsin site mutant is defective in some step subsequent to LF binding[5,6]. Experiments with a PA mutant containing cysteine at the chymotrypsin sensitive site suggested that this region is involved in the translocation of LF into the cytosol. Recently, we found that a PA mutant with an FF314 deletion forms oligomers which are defective for translocation of LF (Singh *et al.*, manuscript submitted).

Table 1. Properties of chymotrypsin-sensitive site mutant PA proteins

Sequence at residues 312-316[a]	Cleavage by proteases (% of control)[b]			Toxicity for RAW264.7 cells[c]
	Tryp	Chymo	Thermo	(EC_{50}, µg/ml)
SFFDI	100	100	100	0.029
SAAAI	100	0	100	0.21
S--DI	100	0	0	>10
SFFAI	100	100	100	0.085
SCFDI	100	5	50	>10

[a]Insequence designations, - - denotes deleted residues
[b]tryp, trypsin; chymo, chymotrypsin and thermo, thermolysin
[c]Toxicity was determined in combination with lethal toxin

The PA protein component of the toxin induces protective immunity against anthrax infection. Purified PA proteins from culture supernatants for use in the vaccines are often degraded due to cleavage by *B. anthracis* proteases. It was anticipated that PA proteins mutated at both the trypsin and chymotrypsin sites would resist proteolytic degradation and could be purified in a more homogeneous form. The double mutant when expressed in *B. anthracis* was much more stable in the culture supernatant. The PA double mutant was non-toxic and may be a useful replacement for PA in anthrax vaccines.

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Editor's note: The following paper invoked lively discussion at the workshop and subsequent WHO anthrax working group meeting. The editor was asked to point out that the outcome of the WHO working group discussion was a recommendation that laboratory workers deriving multiresistant strains should be required to place as much emphasis on the antibiotics they remain sensitive to as on those to which they have become resistant and their susceptibility profiles should, at all times, be as clear as their resistance profiles.

Anthrax prophylaxis by antibiotic resistant strain STI-AR in combination with urgent antibiotic therapy

A. P. POMERANTSEV, YU. V. MOCKOV, L. I. MARININ, A. V. STEPANOV and L. G. PODUNOVA

State Scientific Centre of Applied Microbiology, Obolensk, Moscow Region, 142279, Russia

The incompatibility of live vaccines with antibiotics on simultaneously administration gave rise to the idea of developing a vaccine strain, resistant to antibiotics, that would allow simultaneous prophylaxis and immunization in an emergency. The strain to be used should be resistant to various groups of antibiotics, especially to penicillins and to tetracyclines. These antibiotics are intensively applied in prophylaxis of anthrax infections in Russia.

We have managed to derive a variant of STI-1 strain, STI-AR, resistant to ampicillin (MIC 512 µg/ml), rifampicin (MIC 128 µg/ml), doxycycline (MIC 64 µg/ml), chloramphenicol (MIC 64 µg/ml), macrolides and lincomycin (MIC 128 µg/ml). MIC estimations were carried as follows: each antibiotic was diluted and incorporated into solid media to create a series of plates ranging from 512 µg to 2 µg per ml in two-fold dilutions. The plates were incubated at 37°C overnight and the MICs read as the lowest dilution which showed no bacterial growth.

The resistance to penicillins was induced by means of derepression of *B. anthracis* STI-1 penicillinase gene and a constitutive isolate from this population was characterized. Rifampicin resistant spontaneous mutant strain STI-PR was selected from among the pen^R-population of *B. anthracis* cells and its constitutive resistance to rifampicin was characterized also. Resistance to the remaining antibiotics was obtained by means of insertions of *tet, cat, ermC* genes of different plasmids into the host genome of *B. anthracis* STI-PR strain. Resistance to tetracyclines was only found following induction by the respective antibiotic.

The strain STI-AR retained the normal morphological properties and spore-forming features of the parent STI-1 strain. Resistances to all antibiotics were stable during growth of the STI-AR strain in nutrient media without antibiotic pressure and *in vivo* as well. The experiments on animals (hamsters and rabbits) showed that the STI-AR strain does not differ from its parent strain in residual virulence, safety and immunogenicity. The protective properties of the strain were also studied against challenge with a virulent culture of *B. anthracis* using concurrent doxycyline therapy (Table).

Table: Immunogenenicity of STI-AR strain on the background of infection and special prophylactic treatment with doxicyclin

Animals groups	Survival, %	
	After primary infection and treatment	After second infection
Infection+STI-AR+doxicycline	100	80
Infection+STI-1+doxicycline	100	0
Infection+STI-AR	10	-
Infection+STI-1	10	-
Infection+doxicycline	100	0
Infection (control)	0	0

The results obtained showed that doxycycline prevented the development of infection following exposure to the challenge (Table, column 1) while not interfering with development of immunity in the group vaccinated with STI-AR (80% survival following subsequent exposure - column 2). At the same time all animals given simultaneous administration of STI-1 strain and doxycyline died after the challenge infection. Similar results were obtained with the other antibiotics to which STI-AR was resistant.

Thus, the STI-AR strain is highly resistant to the antibiotics of different classes, retains the immunogenic properties of the initial STI-1 strain and permits immunization in combination with antibiotic therapy on suspicion of exposure to *B. anthracis*.

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Bacillus anthracis cell surface protein antigen with molecular mass of 92 kda plays an essential role in the development of anti-anthrax protective immunity

*BESNOSOV M. V., PETROV G. A. ROGOV S. N., SOROKIN YU. I., PHYLIPPOVA O. L., CHUGAY V. L. BASHKOVA V. A. and EZEPCHUK YU. V.

Anti-Plague Research Institute of Siberia and Far East (Irkutsk), Gamaleya Institute for Epidemiology and Microbiology Russian Academy of Medical Sciences (Moscow)
** (authors unable to attend the workshop)*

Summary

A highly purified protein antigen with molecular mass of 92 kDa was isolated from vegetative cells of *B. anthracis* strain STI-1. The protein was extracted with 3% sodium lauroylsarcosine at 4°C from the intact bacterial cells grown on solid media for 24 h and purified by the absorbtion chromatography on hydroxyapatite procedure. The isolated protein showed homogenicity in SDS-PAGE and Ouchterlony gel-diffusion test with homologous antiserum. When applied in combination with Freund's adjuvant, the protein antigen induced protective antibody in experimental animals and caused an increased phagocytosis response and increased GZT formation. In guinea pigs, two subcutaneous injections of the complex consisting of the isolated protein, PA and EF in the presence of an immunostimulator, resulted in a high level of a specific protection after parenteral challenge with virulent *B. anthracis* spores. These data indicate that the antigen should be considered as an essential component for the future chemical anti-anthrax vaccine.

Development of a *Bacillus subtilis* based system for the expression of the protective antigen of *Bacillus anthracis*

L. W. J. BAILLIE, P. MOORE and R. J. MANCHEE

Microbiology Group, Medical Countermeasures, The Chemical and Biological Defence Establishment, Porton Down, Salisbury, SP4 0JQ, UK

Summary

The protective antigen (PA) of *Bacillus anthracis* is the basis of the current human vaccine. The gene encoding PA expression was transferred into the protease-deficient strain of *Bacillus subtilis*, WB600. PA was produced at levels of approximately 40 mg/L. High level expression occurred in the early logarithmic phase of the culture. The recombinant PA has been characterised and shown to be immunologically similar to PA produced by *B. anthracis*; it invokes a strong immune response in a rabbit model.

Introduction

Bacillus anthracis, the causative agent of anthrax, possesses two known virulence factors; a poly-D-glutamic acid capsule and a tripartite protein toxin (Thorne, 1993). Protective antigen (PA) the non-toxic, cell-binding component of the toxin, is the active protective component of the current human vaccine. The UK vaccine is a protein precipitate from the supernatant of fermenter cultures of the avirulent Sterne strain of *B. anthracis*. This crude vaccine contains, in addition to PA, small amounts of the anthrax toxic moieties, edema factor and lethal factor, and a range of culture-derived proteins. These factors may contribute to adverse reactions to the vaccine in some individuals.

Developments in genetic engineering technology over the past decade should allow the production of the *B. anthracis* PA in a common soil bacterium that does not require high level containment. This approach would also ensure that undesirable components of the anthrax toxin are excluded (Ivins and Welkos, 1986).

Previous workers have attempted to produce PA in *Escherichia coli* (Vodkin & Leppla, 1983) and *Salmonella typhimurium* (Coulson et al, 1994) but for reasons which are not known, the level of production of PA was low in these genetically engineered organisms.

Ivins and Welkos (1986) were the first to report a PA expressing system based on *B. subtilis*. They cloned the gene encoding the protective antigen moiety of the tripartite exotoxin of *B. anthracis* into *B. subtilis* IS53 by using the plasmid vector pUB110. They produced two clones, PA (PA1 [pPA101]) and (PA2 [pPA102]), both of which produced more PA in liquid cultures than the Sterne strain of *B. anthracis* with levels of up to 41.9 mg/l being achieved. The main disadvantage of using *B. subtilis* IS53 (pPA102) was that the organism also produced proteolytic enzymes which degraded the PA and made subsequent purification difficult (Baillie et al, 1994).

We describe here preliminary work directed at developing an expression system based on a proteolytic deficient strain of *B. subtilis* which could produce PA in the quantities required for evaluation as an improved second generation vaccine.

Materials and methods

Bacterial strains and plasmid

B. subtilis WB600 was obtained from Dr Xu-chu Wu, University of Calgary, Canada (Wu et al, 1991). *B. subtilis* IS53 pPA101 and pPA102 were kindly donated by Dr Bruce Ivins USAMRIID, Fredrick, MD, USA (Ivins & Welkos, 1986).

Transformation

B. subtilis WB600 was transformed with pPA101 and pPA102 using the method of Chang and Cohen (1979).

Culture

Unless otherwise stated, all chemicals were obtained from the Sigma Chemical Company (Poole, Dorset, UK). Culture conditions were those described by Ivins and Welkos (1986). Briefly, 250 ml of medium in a 500 ml screw-capped Duran bottle was inoculated with 0.1ml of a suspension of the organism in saline (OD_{540}1.3). Cultures were incubated with shaking (150 rev min^{-1})) at 37°C for 14 h. Growth was monitored by optical density at 540 nm.

For the production of PA the following medium was formulated; 20g Tryptone (Difco Laboratories Ltd., West Molesey, Surrey), 7g K_2HPO4, 3g KH_2PO_4,1g $(NH_4)_2SO_4$, 0.5g sodium citrate, 0.1g $MgSO_4.7H_20$, 2g l-histidine and 1000 ml of deionised water. The pH was adjusted to 7.4 and the medium was sterilised by autoclaving at 115°C for 15 min. Following autoclaving, sterile glucose was added to a concentration of 2%. Finally sterile kanamycin was added to give a final concentration of 10 mg/l.

Determination of PA by ELISA

Solutions were assayed for PA by ELISA (Ivins and Welkos, 1986). Purified PA was obtained from P. Turnbull, CAMR, Porton Down.

Electrophoresis

Samples for SDS polyacrylamide electrophoresis (PAGE), native PAGE and isoelectric focusing were run using the appropriate gels and conditions as detailed by the manufacturer for the PhastSystem™ (Pharmacia Biotech, St Albans, Herts). Gels were stained with either 0.1% Coomassie Blue R-250 and destained with 10% methanol/10% acetic acid/80% deionised water or with silver stain (Heukeshoven & Dernick, 1985).

N terminal sequencing

The N-terminal sequence of the recombinant PA was determined by Dr A. Moir, Department of Molecular Biology and Biotechnology, University of Sheffield.

Biological activity

Recombinant PA was assayed for functional activity in a macrophage lysis assay (Quinn et al, 1991).

Antigenicity of recombinant PA

The binding of a range of antibodies raised against PA produced by *B. anthracis* to recombinant PA was determined by western blot (Baillie et al 1994).

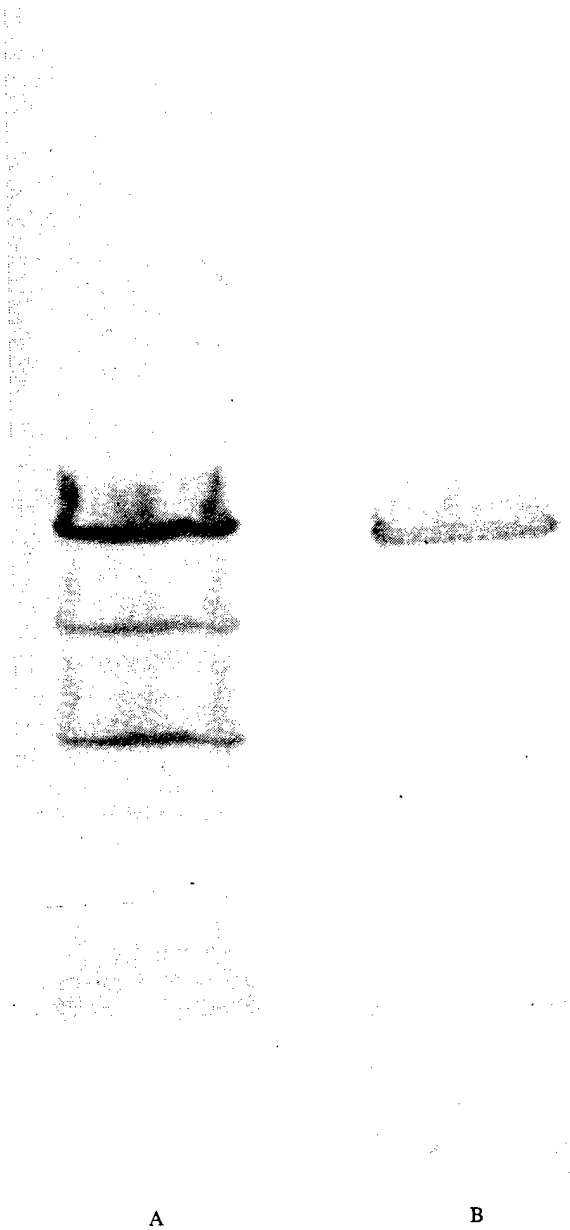
Stimulation of an immune response with recombinant PA

A rabbit was subjected to a series of immunisations with recombinant PA. Each vaccination consisted of approximately 60 µg of purified PA protein resuspended in 500 µl of Freund's incomplete adjuvant and 1.5 ml of distilled water, injected at two different sites on the animal. The animal was vaccinated on days 14, 28, 56 and 84, with a terminal bleed at day 95. The antibody titre was tested qualitatively using the Ouchterlony double diffusion technique. This work was performed by Mr M. Rees at the Department of Microbiology, The Medical School, University of Newcastle-Upon-Tyne.

Results

Expression of recombinant PA

B. subtilis IS53 (pPA101) produced PA at levels up to 40 mg/l. Examination of this PA by western blot showed extensive breakdown of the protein. When the plasmid pPA101 was transferred to the protease deficient strain *B. subtilis* WB600 the resulting construct produced full-size protein at similar levels (Fig.1).



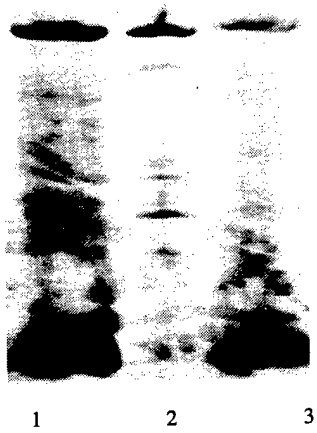
A B

Mature protein

Fig 1. Western blot of culture supernatant of *B. subtilis* IS53 pPA102 (A) and *B. subtilis* WB600 (pPA102) (B) using human anti-PA sera.

Physiochemical characterisation of recombinant PA

Under SDS PAGE electrophoresis conditions recombinant PA from WB600 (pPA101) appeared to migrate the same distance as PA from *B. anthracis* (Fig.2). When recombinant PA was run under non denaturing conditions, the recombinant PA ran as a single band in contrast to PA from *B. anthracis,* which runs as a series of up to 4 charged isomers (Leppla, 1991) all of which migrated further than recombinant PA (Fig.3). Estimation of the pI of recombinant PA by isoelectric focusing gave a value of 6, compared to 5.5 for PA from *B. anthracis.*

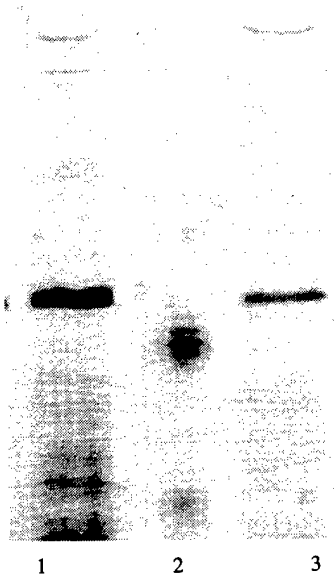


1 2 3

PA

Fig 2. Recombinant and native PA run on a 10-15% gradient PAGE Phast gel in the presence of SDS and stained with Coomassie blue.

Lane 1 = rPA from *B. subtilis* WB600 (pPA101)
Lane 2 = native PA from *B. anthracis* Sterne
Lane 3 = rPA from *B. subtilis* WB600 (pPA102)



1 2 3

PA

Fig 3. Recombinant and native PA run on a 10-15% gradient PAGE Phast gel under non denaturing conditions and stained with Coomassie blue.

Lane 1 = rPA from *B. subtilis* WB600 (pPA101)
Lane 2 = native PA from *B. anthracis* Sterne
Lane 3 = rPA from *B. subtilis* WB600 (pPA102)

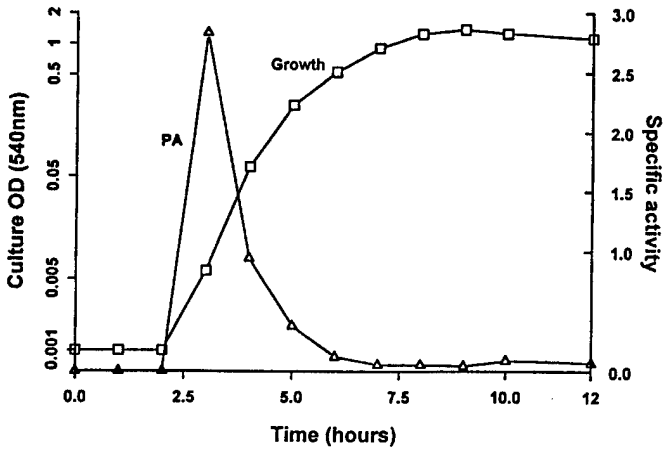


Fig 4. Growth and specific activity of PA from *B. subtilis* WB600 (pPA101)

Antigen recognition

Recombinant PA from WB600 (pPA101) was found to react with polyclonal human and rabbit antisera raised against the current PA vaccine. In addition it recognised two distinct monoclonal antibodies, C3 (R. Bohm, University of Hohenheim, Stuttgart, Germany) and 3B6 (Little et al, 1988) both of which are specific for PA from *B. anthracis*.

N-terminal sequence

The first ten amino acids of the recombinant PA from WB600 (pPA101) were EVKQENRLLN, identical to the published sequence for the N-terminus of the mature protein (Welkos et al, 1988).

Biological activity

Recombinant PA produced by WB600(pPA101) was biologically active. The level of activity, 42 mg/L, was similar to the levels of PA attained following culture of this strain as determined by ELISA.

Immunogenicity

Recombinant PA from WB600(pPA101) invoked a strong immune response at day 66 in the vaccinated rabbit. This response was stronger than the response seen with PA from *B. anthracis*.

Time course of PA expression

The specific activity (mean ELISA-determined PA absorbance, A_{412}, per culture optical density unit, OD_{540}) of PA expression from *B. subtilis* WB600 (pPA101) is shown in Fig. 4. A similar pattern of expression was seen with *B. subtilis* IS53 (pPA101).

Discussion

To overcome the problem of degradation of recombinant PA by host-produced proteases, the gene encoding PA production was transferred into the protease-deficient strain *B. subtilis* WB600. This organism has been engineered to be deficient in six extracellular proteases (Wu et al, 1991). This strain produced 40 mg/l of full size recombinant PA and should allow the development of a purification strategy.

Characterisation of the recombinant PA showed that it differs in some aspects from *B. anthracis* PA. These differences may be due to some form of post translational modification (Creighton, 1993) or to alterations to the gene itself.

While the recombinant PA differed in some respects to PA from *B. anthracis*, it showed antigenic similarities. Ivins found that the PA from *B. anthracis* Sterne (pPA102) was able to invoke protection against aerosol challenge with spores of *B. anthracis* in an animal model (Ivins et al, 1994). Given the similarity between the two proteins, and the fact that a strong immune response was invoked with recombinant PA, there is a good probability that the recombinant PA will be protective. Further work is required to determine whether recombinant PA will protect against aerosol challenge with spores of *B. anthracis*.

High level PA expression by WB600 occurred in the early logarithmic growth phase; down regulation began as the cells progressed further into log phase. This was seen with both WB600 and IS53. It was expected that strong expression of PA in *B. subtilis* would occur throughout the vegetative growth phase as the P2 promoter of PA closely resemble the consensus sequence for recognition by the primary house-keeping sigma factor of *B. subtilis* RNA polymerase (Moran, 1989).

Down-regulation early in growth suggests either the involvement of a limiting factor such as a medium component, or the presence of a negative regulator. By elucidating the nature of this regulation of PA expression it may be possible to increase the level of PA expression.

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Bacillus anthracis: an expression vector for *in vivo* delivery of antigens

JEAN-CLAUDE SIRARD, CORINNE PEZARD, EDITH DUFLOT, MARTINE WEBER and MICHELE MOCK

Laboratoire de Génétique Moleculaire des Toxines, Institut Pasteur, 28, rue du docteur Roux 75724 Paris Cedex 15, France

Introduction

The two toxins of *Bacillus anthracis* are composed of the binary combinations of three proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF). The *B. anthracis* Sterne strain, which contains the pXO1 virulence plasmid and which is therefore toxinogenic, is able to induce protective immunity against anthrax in many animal species. Mutant strains of the Sterne strain that are deficient in the production of one or two of these toxin components have been previously constructed[3]. In this work, we examined, on the one hand, the protection conferred by these strains against experimental anthrax and, on the other hand, the mechanisms involved in the immunity.

We also present data on the use of *B. anthracis* as an expression vector for *in vivo* delivery of heterologous antigens.

Materials and Methods

B. anthracis strains used in this work have been previously described[1, 3, 4]. 7702 is the parental Sterne strain and the plasmidless strain 7700 was derived by curing 7702 of pXO1. The single mutant strains RP9 and RP10 produce PA + LF, and PA + EF, respectively. The double mutant strains RP42, RP31, and RP4 produce PA, EF, LF, respectively. Only RP9 is still lethal with an LD_{50} of 10^7 spores, which is twenty-fold more than that of Sterne strain[3].

Female, six-week-old, pathogen-free Swiss mice (Iffa Credo, France) were immunized subcutaneously with a single dose of spores (10^6, 10^7 or 10^8 spores per mice) of the appropriate *B. anthracis* strain. Six weeks after immunization, mice were challenged subcutaneously with 1,000 times the LD_{50} of the Sterne strain.

For serological studies, mice (10 per group or, 20 to 30 per group for RP9) were infected with approximately $5x10^7$-10^8 spores of the appropriate *B. anthracis* strain. Six weeks after immunization, antibody titers to PA, LF, or EF, or to extracellular antigens (EA), were determined by an enzyme-linked immunosorbent assay (ELISA). The EA preparation represents crude material released by strain 7700 into the medium[5].

Results and discussion

A new veterinary vaccine against anthrax

The requirements for a bacterium to be a good live vaccine are summarized in Table 1.

Table 1. Requirements for a live bacterial vaccine

Characteristics required	Comments
avirulent	no lethality nor side effects
development *in vivo*	production of protective antigens
persistence *in vivo*	long-lasting immunity
stable strains	genetic; storage

As expected, the Sterne strain (7702) has some of the required characteristics but needed nevertheless to be improved in regard to its residual virulence. For this purpose, we studied in a mouse model the behavior of six toxin-deficient strains of *B. anthracis*[3, 4]. First, the virulence of these recombinant strains was analyzed and compared to that of Sterne strain and a non-toxinogenic strain cured of pXO1. All the strains were avirulent except RP9, the lethal toxin producing strain, which had reduced virulence. The six toxin-deficient strains could therefore represent safer live vaccines than the Sterne strain.

The *in vivo* development of all strains was monitored by following the antibody response to extracellular antigens (EA) of vegetative bacilli. Only $pXO1^+$ strains elicited antibodies against EA (Table 2)[5]. These results suggest that pXO1 is required for *in vivo* development of spores; it probably encodes factors involved in either germination or multiplication, or resistance to host defences. Moreover, bacteria of pXO1+ strains persisted longer than those of the pX01-cured strain at the site of injection. These two characteristics are also required for efficacy of live vaccines, suggesting that the six toxin-deficient strains are potentially good candidates.

Table 2. Antibody response to vegetative extracellular antigens in mice immunized with *B. anthracis* toxin-deficient strains

Mutant Strains	ELISA antibody titer to extracellular antigens*
pXO1-cured	50
$pXO1^+$ (RP4, RP8, RP9, RP10, RP31, RP42)	1600

* Reciprocal geometric mean titer. For $pXO1^+$ strains, the arithmetic mean of reciprocal titer is given.

In a preliminary approach, the mutant strains were assayed for their protective immunity against a lethal challenge with the Sterne strain[5]. It appeared that all strains containing pXO1 protect to a certain extent against anthrax. These results suggest that pXO1-encoded factors other than toxins are required for protection in mice and they are in agreement with the differences observed in bacterial development and persistence. Moreover, at low immunizing doses (10^6 spores per mice), PA producing strains appeared significantly more protective than other strains. These data support the idea that PA is an important protective antigen.

The antibody response against the toxin components produced *in vivo* by the mutant strains was also studied. All PA-producing strains induced a strong antibody response against PA (ELISA titer of 1:3913). In contrast, the response against EF or LF was significantly lower in PA-deficient (1:137 and 1:204 respectively) than in PA-producing strains (1:662 and 1:3919 respectively). This adjuvant effect of PA on the antibody response to EF or LF probably reflects a different processing and presentation of these two antigens in the presence of PA. It is interesting to note that the best protection is obtained with lethal toxin or edema toxin producing strains for which the antibody titers are high for all toxin components. Therefore, in PA producing strain, the contribution of EF and

LF in protective immunity is not negligible and could be mediated by the humoral response.

A future vaccine against anthrax could certainly be designed considering these new data. In other words, the efficacy of the safe, toxin-deficient strains (mainly the edema toxin and the lethal toxin producing strains) should now be assayed using a challenge with a virulent strain in the guinea-pig (used for anthrax vaccine trials) and the cattle.

Towards the use of B. anthracis as an in vivo expression vector

B. anthracis Sterne strain and its derivatives produce large quantities of toxin components both *in vitro* and *in vivo*. It has also been shown that the expression of toxin genes is regulated at the transcriptional level. These characteristics suggest that *B. anthracis* could be a potential expression vector for heterologous antigens.

We decided to assay the system using the iota toxin Ib component of *Clostridium perfringens*[2]. The Ib protein (B component of the toxin) was chosen for several reasons: (i) Ib is a gram-positive protein, (ii) the iota toxin is responsible for a veterinary disease and mediates the main physiopathological effects, (iii) the protective immunity to toxinogenic bacteria is usually conferred by neutralizing antibodies against the B component, and (iv) Ib shows similarities with PA.

A fusion between the *pag* regulatory region (*pag* encodes PA) and the Ib structural gene was constructed in *Escherichia coli* and transferred in the LF-deficient *B. anthracis* strain RP10. The resulting recombinant strain is stable and has integrated the *pag-ibp* fusion at the *pag* locus on pXO1. Expression *in vitro* and *in vivo* in the *ibp* gene in *B. anthracis* is currently being measured.

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Comparison between protection by anthrax vaccines and infection by *Bacillus anthracis* field strain in Brazil

* R. D. NEVES[1], R. CORSEUIL[1], C. PIANTA[2] and SANTOS, H. T.[3]

[1] Ministry of Agriculture, Porto Alegre, Brasil. [2] Centro de Pesquisas Veterinarias "Desiderio Finamor", CP. 2076 CEP 90001, 970, Porto Alegre, RS, Brasil, [3]State Veterinary Office, Uruguaiana-RS, Brasil

** (Authors unable to attend theworkshop)*

The municipality of Uruguaiana is situated in what was, in the past decades, an endemic area of anthrax. This paper describes an outbreak of the disease in bovines found either sick or dead near a place formerly used to shear sheep.

Fifteen cattle, five sheep and three horses died from *B. anthracis* infection; the clinical signs, necropsy findings and bacteriological culture led this conclusion. All these animals had been vaccinated against anthrax.

Six commercial anthrax vaccines produced by five different industries, kept under good storage condition and within the validity period were purchased and tested for viability, lack of virulence, purity of culture, phage sensitivity and hemolysis production on blood agar plates after 24 h incubation at 37°C.

Twenty-one sheep were used in groups of three. Each group received a single dose of vaccine. One group (3 animals) was not vaccinated and was kept together with the 28 vaccinated sheep (Corriedale adult females).

Twenty-nine days after the vaccination, the animals were challenged with *B. anthracis* strain "C" isolated from a dead horse. The LD_{50} was determined by the Reed-Muench method, and 1000 guinea-pig doses were inoculated into each sheep. The animals were observed daily for up to 15 days after inoculation.

The phage typing done with gamma phage and the hemolysis production showed that the isolate from culture of one vaccine was not susceptible to phage while that from another other vaccine sample grew hemolytic colonies. The vaccines used in these two groups did not protected the animals and the sheep died after the challenge with the strain "C". One vaccine protected 2 out of 3 animals in the group. Only 3 of 6 commercial anthrax vaccines protected all the vaccinated animals.

The vaccine used four times in the farm, showing no protection, was the same product which showed no phage susceptibility and a large hemolysis in the blood agar medium. The analysis of the results allow the conclusion that the lack of protection after the vaccination was mainly due to failure to apply proper quality control on the part of the manufacturers of the vaccines concerned.

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Antimicrobial susceptibility of *Bacillus anthracis* against macrolides

B. SUMERKAN[1], B. AYGEN[2], M. DOGANAY[2] and E. SEHMEN[2]

[1]Department of Microbiology and [2]Department of Infectious Diseases, Faculty of Medicine, Erciyes University, 38039 Kayseri-Turkey

Penicillin is the drug of choice in the treatment of anthrax although limited number isolates of penicillin-resistant *Bacillus anthracis* have been reported[1,2]. Alternative drugs in penicillin sensitive patients are erythromycin, tetracycline and chloramphenicol[3]. New macrolides (azithromycin, clarithromycin and roxithromycin) have been introduced in medical therapy in recent years. In vitro activity of these antibiotics against *B. anthracis* has not been tested. The purpose of this study was to determine the susceptibility of *B. anthracis* against macrolides.

Materials and methods

This study included 34 isolates collected from the same region between 1983 and 1994. Azithromycin (Pfizer), erythromycin (Fako), clarithromycin (Abbott) and roxithromycin (Turk Hoechst) were obtained as sterile powder.

All strains were plated for purity on blood agar and incubated at 37°C overnight. Bacterial suspensions were prepared from the colonies in sterile saline and the densities adjusted to 0.5 McFarland standard. This suspension was diluted 1/100 in sterile saline to a concentration of 10^6 cfu/ml. A micropipette which transferred 10 µl volumes was used to inoculate the plates to achieve a final inoculum of 10^4 cfu per spot.

The minimum inhibitory concentrations (MICs) of each antibiotic were determined by an agar dilution technique[4]. Twofold serial dilutions of antibiotics in Mueller-Hinton agar (BBL) yielded concentration ranges for the antibiotic of 64 to 0.03 µg/l. Another plate without antibiotic was used as a control. *Staphylococcus aureus* (ATCC 29213) was used as the control microorganism. The MIC was read as the lowest dilution which showed no bacterial growth.

Results and discussion

The MIC results for erythromycin, azithromycin, clarithromycin and roxithromycin are shown in Table 1. All the isolates were sensitive to erythromycin, with MIC of 0.25-1 µg/ml.

New macrolides showed good activity against all strains of *B. anthracis*. The MIC values were between 0.5-4 ug/ml in azithromycin, 0.03-0.25 µg/ml in clarithromycin and 0.06-0.25 µg/ml in roxithromycin.

Tetracycline and erythromycin are alternative drugs in the treatment of patients who are hypersensitive to penicillin or infected with one of the rare penicillin-resistant strains[2,3]. Excellent results in the treatment of cutaneous anthrax had been noted with chlortetracycline, erythromycin, tetracycline, oxytetracycline and streptomycin[3] No resistance to erythromycin was found in previous studies[1,5,6].

New macrolides tested showed a good activity against all strains of *B. anthracis* in this study. These agents should be considered as alternative therapies for less severe cutaneous anthrax.

Table 1. *In vitro* activity of macrolides against *Bacillus anthracis*

Macrolide	Minimum Inhibitory Concentration (µg/ml)		
	Range	50%	90%
Azithromycin	0.5-4	1	4
Clarithromycin	0.03-0.25	0.06	0.12
Erythromycin	0.25-1	0.5	1
Roxithromycin	0.06-0.25	0.25	0.25

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Bits and pieces (from the poster boards at the workshop)

TOPIC: Incubation period

E. Shlyakhov writes: Data from my card index covering 1215 cases of acute cutaneous anthrax in Moldavia, 1946-50, indicates an average incubation period of 2-3 days, range 9 h to 10 days.

In the case with a 9 h incubation period, a housewife had beaten an old sheepskin coat at about 10h00 and wisps of the coat fell on her face. At about 19h00, she noted an itching papule on her chin, which subsequently developed into a classic anthrax carbuncle.

A Russian author, I.A. Dmitriev, published a story in 1929 of an accidental anthrax outbreak in an anti-rabies unit at Kursk, in central Russia. Of 65 patients receiving anti-rabies vaccine from a syringe previously used for dispensing anthrax spores, 30 developed cutaneous anthrax with incubation periods of 1 day (1 patient), 3 day (2 patients), 4 days (2 patients), 5 days (2 patients), 6 days (10 patients), 7 days (5 patients), 9 days (1 patient), and 12 days (1 patient), with the time of onset not recorded for the remainder.

Ed adds: With reference to Professor Shlyakhov's case of the housewife with a 9 h incubation, DE Salmon (of *Salmonella* fame) recorded in 1896 personally seeing an anthrax pustule develop on the knuckle of a groom 12 h after using a new horse brush (Salmon DE. Diseases of the horse. USDA, Government Printing Office, Washington 1896; 528).

M. Doganay writes: From our files, the incubation period appears to be 3 to 5 days with a range of 2 to 12 days.

R. Pfisterer writes: I refer to my article in the *Schweiz med Wsch* 1991; **121**: 813-25. In our epidemic, we determined the incubation time with absolute accuracy in 9 cutaneous cases to be 2.5 to 5 days, mostly 3 to 4 days.

Other references: Bouquien (Sur une épidémie de charbon animal et humain observée dans le Morbihan, Bull Acad Nat de Med 1950; **134**: 585) reports 2 to 5 days; Gold (Anthrax - a report of 117 cases, Am Med Ass Arch Int Med 1955; **96**: 387) gives 12 h to 5 days, average 3 days; Brachman *et al* (An epidemic of inhalation anthrax, Am J Hyg 1960; **72**: 6) says 1-8 days.

TOPIC: References to penicillin resistance

British case: A 53-year old clerk in an engineering firm. Garden bonemeal suspected as the source of infection. *Br Med J* 1976, i: 748.

France: According to GHB Martin (MD thesis, Dundee, 1975), penicillin resistance was reported by Chambon and Dutrenit, *Bull Soc Pathol Exotique* 1955; **48**: 546.

Bosnia: 38-year old woman, following a gad-fly bite. *Lancet* 1992; **340**: 306-7.

Japan: Riyugasaki strain, from a cow. MIC 8000 u/ml. (Koshimizu *et al, Jap Vet Soc J* 1972; 25: 184-9).

TOPIC: Second attacks

E. Shlyakhov writes: I observed 3 cases of a second cutaneous anthrax infection. These occurred 8, 15 and 20 years, respectively, after the first attack. Two of the patients were veterinarians; in one of them, the carbuncle was localised in the same place on the arm as 15 years previously.

GHB Martin, MD thesis (Dundee) 1975: "Reinfection of the skin was seen not infrequently at Rassa (Ethiopia), and the second lesion was usually noted to be less severe than the initial one. This accords with the observations of Sinderson (*Br Med J* 1933; **i**: 612) and Hodgson (*Lancet* 1941; **i**: 811)." [Hodgson's case was in a veterinarian who was infected on 3 occasions].

TOPIC: Mortality and untreated cutaneous anthrax

In Britain, in the 13-year period, 1899 through 1912, before antibiotics and vaccines were available, of 315 cases of industrial cutaneous anthrax, 40 (12.5%) were fatal (Report of the British Departmental Committee, HMSO Cd. 9172, 1918).

E. Shlyakhov writes: In 622 cases with anthrax lesions on the head and face, 52 (8.2%) died. In 181 cases with anthrax on the neck or back of the head, 14 (7.7%) patients died. In 357 cases with carbuncles on upper extremities, 11 (3.1%) patients died, and in 34 cases with the lesions on the lower extremities, 1 (2.9%) patient died. No fatalities occurred among 17 cases where the lesions were on the trunk. The most fatal localisations were eyelids and chins (>50% lethality).

TOPIC: History. Setting the record straight.

R. Pfisterer writes: "In every historical review published on anthrax, it is said that the word "anthrax" was used by Homer and Hippocrates in reference to a disease. Close examination of the original texts shows that Homer never used the word "anthrax" in reference to a disease. This word can only be found once in the works of Homer (Ilias **IX**: 213) and means live coals. In the Hippocratic texts, "anthrax" also means, with few exceptions, live coals. In some of these texts, "anthrax" means varied, non-specific, vesicular or pustular dermatitits without any indication of contact with diseased animals or of epidemics. The differentiation between "benign anthrax" and"malign anthrax" was only made much later.

TOPIC: Where is the Ascoli test still used?

Korea; rabbit antiserum used (*Han Sang Yoo*)

Several countries of Europe (*R. Böhm*)

TOPIC: Where apart from China is serum therapy still used?

Russia (*B.L. Cherkasskiy*)

TOPIC: Recalling an outbreak of gastrointestinal anthrax in northern Kenya, 1996. *(Dr C. van den Bosch, Dept of Public Health, East London & The City, Bow House, 153 Bow Road, London E3 2SE)*

A group of villagers in the Meru District, Kenya, disposed of a cow that had sickened and died by eating it. The following day, they became ill with diarrhoea and approximately 12 of those afflicted were conveyed to the local hospital. Their chauffeur, a Missionary, also brought with him the spleen of the dead animal. The patients ranged in age from children to one frail elderly lady, possibly around 70 years old. They were all dehydrated and prostrate with high pyrexias,ranging from 102°-106°F. The majority had temperatures around 103°-104 °C. The patients were treated with oral chloramphenicol for 7-10 days and fluids, given intravenously where necessary. All the patients recovered with the exception of the old lady, with a temperature of 106 °C, who died within 48 h of admission. A Gram stain of a smear impression of the spleen of the cow revealed typical boxcar-shaped, Gram-positive rods.

TOPIC: Age/sex susceptibility differences

A reference you may not find in your conventional literature search is that of GHB Martin, MD Thesis (Dundee) 1975 "Cutaneous anthrax in rural Ethiopia: a study of one hundred consecutive cases; their clinical features and epidemiology". In relation to age/sex differences, he states "Many cases occurred in chilfdren under 10 (31 cases), and in young males, (28 male patients were in the decade 15 to 24 years). The disease presented infrequently in patients over 30 years of age (less than 20% of patients) and in females over 10 years old (17% of patients).